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PhD Thesis

Nanohydroxyapatite-based antibacterial surfaces to prevent biofilm associated biomaterials bone infection

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ABSTRACT

In orthopaedics, as in many other medical fields, the use of implants has greatly improved the quality of life for an increasing number of patients. Nowadays, orthopaedic implants are used routinely worldwide for fixation of long bone fractures and non-union defects, for correction and stabilization of spinal fractures and deformities, for joint replacement, and for other orthopaedic and maxillofacial applications. Despite the rapid evolution of implant technologies and bone grafting techniques, there is still a great demand for novel and more sophisticated synthetic materials for bone applications. Among biomaterials used for bone-related applications, hydroxyapatite (HA) has received considerable attention due to its excellent bioactive and osteoconductive properties as it bonds to bone and enhances bone tissue formation. In particular, synthetic HA with crystals within the nanometer range (nanoHA) has superior functional properties due to its biomimetic chemistry and morphology when compared to the mineral phase of bone. However, adhesion of microorganisms on biomaterials with subsequent formation of antibiotic-resistant biofilms is a critical factor in implant-related infections and it is currently regarded as the most severe and devastating complication associated to the use of biomaterials. In this context, the main purpose of this work was the development of nanoHA based-anti-infective biomaterials to prevent or treat implant colonisation and, subsequent biofilm formation. To address this goal both inorganic and organic approaches were investigated. Initially a composite that combines the favourable biological characteristics of nanoHA and, simultaneously, possesses antimicrobial activity as expressed by ZnO was synthesized as dense discs and the primary objective was to determine whether the size of ZnO particles (from the micrometer scale down to the nanometer range), when incorporated into nanoHA, was playing an important role in inhibiting bacterial growth. The materials had robust *in vitro* antibacterial activity, with an inversely proportional relationship between particle size and antibacterial effect. Accordingly, the challenge of the following step was the continued research on nanoHA-ZnO composites but this time for the production of three-dimensional and interconnected porous granules. The experimental evidence supports the view that nanoHA-ZnO granules exhibited antibacterial activity not only *in vitro* but also *in vivo*, and the interconnected porous scaffolds support tissue recovery, with the presence of blood vessels and tissue regeneration *in vivo*. Regarding the

organic approaches, two bee-derived natural extracts, namely green and red propolis, were adsorbed on nanoHA substrates and their antibacterial effectiveness was observed through the reduction of bacterial growth and biofilm formation while being non-cytotoxic to fibroblast cells. In a slightly different approach, the activity of three novel imidazole derivatives to prevent *Candida* spp biofilm formation as well as to eradicate pre-formed *Candida* spp biofilms on nanoHA substrate was studied. The antifungal agents displayed strong inhibitory effect on biofilm development as potent *in vitro* activity against sessile cells within biofilm. Overall, this work reached the aimed results and presents promising strategies that could improve the capacity to prevent or eradicate biofilms in medical devices. Nevertheless, comparing the results obtained with the various materials tested in this work, nanoHA-ZnO porous granules take the best position as candidates for translational research from benchside to bedside.

RESUMO

A utilização de implantes médicos no campo da ortopedia permite melhorar substancialmente a qualidade de vida de um número cada vez maior de pacientes. Estes implantes são atualmente usados para fixação de fraturas ou defeitos ósseos, para correção e estabilização de fraturas na coluna vertebral, para substituição de articulações danificadas, entre outras aplicações ortopédicas e maxilo-faciais. Apesar do rápido desenvolvimento na área dos implantes e enxertos ósseos, continua a existir uma grande necessidade de novos e mais sofisticados biomateriais sintéticos. De todos os biomateriais utilizados em aplicações relacionadas com o osso, a hidroxiapatite (HA) atrai enorme atenção devido à sua excelente bioatividade e osseocondutividade, uma vez que se liga ao osso e conduz à formação de novo tecido ósseo. Em particular a HA nanométrica (nanoHA) apresenta propriedades funcionais superiores, dada a sua semelhança química e morfológica com a fase inorgânica do osso. Contudo, a adesão de microrganismos aos biomateriais com a subsequente formação de biofilmes, altamente refratários aos antibióticos, é um aspeto crucial nas infeções relacionadas com implantes médicos, e uma das complicações mais severas e devastadoras associadas ao uso clínico dos biomateriais. Neste contexto, o objetivo deste trabalho centrou-se no desenvolvimento de biomateriais resistentes à infeção baseados em nanoHA, para prevenir ou tratar a colonização microbiana e subsequente formação de biofilme. Para atingir este objetivo foram avaliados compostos orgânicos e inorgânicos. Inicialmente foram sintetizados compósitos que combinaram as características favoráveis da nanoHA com a intrínseca atividade antimicrobiana do óxido de zinco (ZnO). Numa primeira etapa avaliou-se a influência do tamanho das partículas de ZnO na inibição do crescimento bacteriano. Os materiais mostraram forte atividade antibacteriana *in vitro* sendo esta inversamente proporcional ao tamanho da partícula de ZnO. Numa segunda etapa sintetizaram-se estruturas tridimensionais, nomeadamente grânulos de nanoHA-ZnO. Os resultados obtidos permitiram inferir que tais materiais mantêm atividade antibacteriana não apenas *in vitro*, mas também *in vivo*. A estrutura porosa, altamente interconectada, dos grânulos permitiu o crescimento de tecido *in vivo* e neovascularização. Relativamente às abordagens orgânicas, dois extratos naturais, nomeadamente própolis verde e vermelha, foram adsorvidos aos materiais de nanoHA. Os materiais resultantes mostraram-se eficazes na redução do crescimento bacteriano

bem como na inibição da formação de biofilme, mantendo citocompatibilidade quando incubados com fibroblastos. Numa abordagem ligeiramente diferente, a atividade de três novos compostos derivados do imidazole foram testados quanto à sua capacidade de prevenir a formação de biofilme por *Candida* spp bem como à sua capacidade de eliminação de biofilmes pré-formados na superfície de materiais de nanoHA. Os compostos apresentaram forte atividade antifúngica tanto no desenvolvimento do biofilme quanto em células embebidas no biofilme. Em termos gerais este trabalho apresenta abordagens promissoras que podem melhorar a capacidade de prevenção ou tratamento de biofilmes associados ao uso de biomateriais. De todas as abordagens testadas, os grânulos de nanoHA-ZnO foram aqueles que apresentaram maior potencial para a translação desde o estudo de bancada até ao paciente visando uma mais direta aplicação clínica.

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CHAPTER I

Introduction

1. Bone tissue engineering

Bone is a remarkable organ playing key roles in critical functions in human physiology. The importance of bone becomes even clearer in the case of diseases such as osteogenesis imperfecta, osteoarthritis, osteomyelitis, and osteoporosis where bone does not perform adequately. These diseases along with traumatic injury, orthopaedic surgeries (i.e., total joint arthroplasty, spine arthrodesis, implant fixation) and primary tumour resection lead to or induce bone defects or voids [1, 2]. The high incidence of these conditions is evidenced by the 2.2 million bone grafts used annually worldwide in order to repair bone defects in orthopaedics, neurosurgery and dentistry. Therefore, the clinical and economic impact of bone defects treatments is staggering [3].

In view of these clinical events the ways to heal the impaired bone tissue in timely manner can be regarded as one of the most important tasks that biomedical engineering can contribute to. Implant technology is developing rapidly however, there is a significant proportion of patients with bone defects or non-unions impossible to healing by direct fixation alone. Consequently, there is a great demand for novel bone replacement and bone regeneration systems beyond traditional surgical strategies. Historically, the 'gold standard' treatment is autogenous bone grafting. In this case, bone tissue is transplanted from one site to another in the same patient, ensuring the histocompatibility and non-immunogenicity. However, harvesting autografts is expensive, painful, constrained by anatomical limitations and associated with donor-site morbidity due to infection and hematoma [4-7]. Another reasonable option for patients and surgeons is the use of bone tissue from one individual to another called allograft. Allografts can be collected from either living donors or non-living donors and must be processed within a bone tissue biobank [8-11]. Allografts offer the advantage of allowing the surgeon to place a graft of the same anatomic location, and thus with very similar mechanical and biochemical properties, as the recipient site [12]. However, allografts carry risks of donor to recipient infection (rate of incidence of 5.4%), disease transmission, and host immune responses along with possible graft rejection [13, 14]. Although both these types of bone grafts have been widely used, there is a strong motivation on the development of novel and more sophisticated synthetic materials, tissue-engineered, to repair segmental defects caused by the removal of infected tissue or bone tumours or to improve the bone healing response and regeneration of bone

tissue around surgically implanted devices, such as artificial joints replacements or plates and screws used to keep bone alignment.

The fundamental concept behind tissue engineering is to utilize the body's natural biological response to tissue damage in conjunction with engineering principles. Thus, tissue engineering can be defined as an interdisciplinary field that applies the principles of engineering and life sciences to develop biological substitutes [15]. Several major technical advances have been achieved in the field of bone tissue engineering during the past decade, especially with the increased understanding of bone healing at the molecular and cellular level. As a result, biomedical engineers have been developing a wide variety of synthetic substitutes which serve as two- or three-dimensional templates to restore, maintain or improve tissue function [7, 15]. The use of such synthetic biomaterials also allows overcoming the main drawbacks of autograft and allograft procedures, including the elimination of disease transmission risk, fewer surgical procedures, a reduced risk of infection or immunogenicity, and the abundant availability of synthetic materials.

What separates a biomaterial from all others is its ability to exist in contact with tissues of the human body without eliciting an unacceptable degree of harm to that body [16]. In bone tissue engineering, the first challenge lies in the selection and optimization of the 'best' biomaterial for medical applications that mimics native bone tissue [1]. Such ideal biomaterial is a matrix that acts as a temporary substrate allowing cell growth and tissue development. The biomaterial should be able to mimic the structure and biological function of the native extracellular matrix (ECM) in terms of both chemical composition and physical structure, and not evoke any adverse or excessive inflammatory response [2]. To address these physiochemical biomimetic requirements specifically, a synthetic bone scaffold or graft must provide appropriate mechanical support to the affected area, contain a porous architecture to encourage mesenchymal stems cells (MSCs) migration and grow on its surface (osteoconduction), support and promote osteogenic differentiation of MSCs (osteoinduction) and enhance production of ECM by osteoblastic cells present within the graft material (osteogenesis) towards biomaterial-host tissue integration (osteointegration), without an intervening layer of fibrous tissue [17-19]. Other properties that must also be addressed comprise degradation in a controlled manner to facilitate load transfer to developing bone,

produce non-toxic degradation products, not incite an active chronic inflammatory response, be capable of sterilization without loss of bioactivity, easy to use, and cost-effective. Additionally, they can also deliver bioactive molecules or drugs in a controlled manner to accelerate healing and prevent pathology [1].

To fulfil all these requirements, a clear concept of bone biology, physiology, and anatomy is, therefore, essential.

1.1. Bone properties

Bone is a highly vascular mineralized connective tissue which plays a wide variety of critical roles in human physiology. Bones not only provide the basic mechanical support to the body by generating and transferring forces that are involved in locomotion, but bones such as skull or ribs, also serve to physically protect vital internal organs including brain, heart, and lungs. Additionally, bone is the main mineral reservoir for the body by storing minerals within, mostly calcium and phosphate but also many other ions, and also stores growth factors, fatty acids, heavy metals, and other toxic elements. Absorption and release of salts is the mechanism by which bones buffer the blood and prevent excessive pH changes. Some bones are multiple progenitor cell (mesenchymal, hematopoietic) housing [1, 20].

From the biological perspective natural bone is a porous nanocomposite material made up of inorganic components, primarily hydroxyapatite (HA), a calcium phosphate material, and a large diversity of organic components, mostly collagen fibrils, but also an abundance of other non-collagenous proteins and a minor amounts of lipids and osteogenic factors [1, 21, 22]. The mechanical strength of the composite comes from the inorganic HA, which, combined with type I collagen fibres and the rest of ECM, is able to provide a supportive scaffold. The nanoscale HA crystals are responsible for imparting an appropriate compressive strength, whereby collagen fibres, able to dissipate energy effectively, provide superior elastic properties, thus improving the mechanical behaviour of HA itself. Thus, the two components of bone enable it to be strong yet allow some elastic deformation [1, 21, 23]. However, the exceptional strength and toughness of bone derive not only from the synergetic combination of its mineral

and organic components, but also from its hierarchical, superstructural organization (Figure 1) [24].

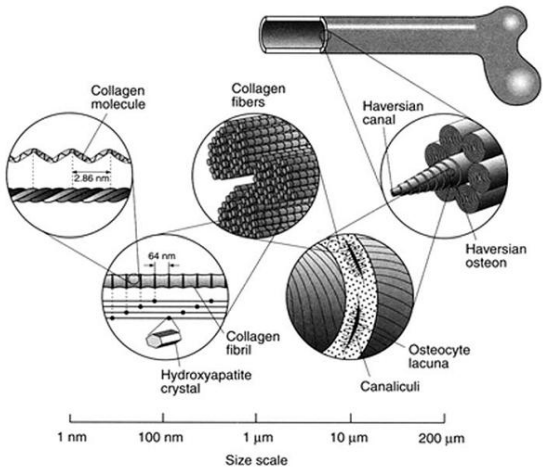

Figure 1.Bone is a complex, hierarchically structured biological material that comprises macro, micro and nano components. The microstructure of cortical bone consists of osteons with Haversian canals and lamellae, and at the nanoscale, the structural units are collagen fibers composed of bundles of mineralized collagen fibrils with hydroxyapatite crystals (adapted from [20]).

Further to its mineral and protein components, bone is also populated by cells, macromolecules, and blood vessels. To maintain a healthy regenerative bone structure, three cell types are involved, as follows: osteocytes involved in signal transduction of mechanical stimuli; osteoblasts which are differentiated from MSCs and whose primary function is to synthesize and secrete organic matrix and lay down pre-mineralized bone matrix, i.e., osteoid; and osteoclasts which are multinucleated cell derived from fusion of mononuclear hematopoietic precursors and whose primary role is bone resorption through the secretion of acids and proteolytic enzymes, that degrade the mineralized tissue under the influence of chemical cues [25, 26]. Osteoblasts may have two fates. They can either become embedded in their own bone matrix as osteocytes or undergo apoptosis (programmed cell death) [25].

Through the cooperative action of osteoblasts and osteoclasts, bone ECM is constantly being remodeled in response to physiological requirements [27]. Bone remodeling is a critical process that takes place during the organism’s lifetime and it accounts for its growth during the development stages of the body, for maintaining skeletal integrity, healing, blood calcium regulation, and accommodation of changes in

bone stress profiles. It is a complex process that occurs in three distinct phases: resorption (osteoclasts are activated through paracrine pathways to digest bone), reversal (mononuclear cells appear on the surface), and formation (osteoblasts produce and secrete ECM). Bone remodeling is responsible for adjusting the architecture and hence mechanical properties of bone as a function of mechanical and chemical signalling [28, 29].

One very important regulator in bone physiology, especially bone remodelling, is mechanical stimulation and cellular transduction. Bone cells are profoundly influenced by the loads that they experience *in vivo*. During skeletal movement, forces are transmitted to cells via direct strain, hydrostatic pressure, flow-induced shear and electric fields. Although osteocytes are generally considered to be the primary sensing cell, *in vitro* studies have shown that osteoblasts, osteoclasts and osteoprogenitor cells also respond to mechanical stimulation [30, 31]. Additionally, chemical cues also play a significant role in basic cell functionality such as migration, adhesion, proliferation, differentiation and ECM production. In all tissues, the extracellular environment contains a wide variety of molecular signal which can be found in immobile, insoluble hydrated macromolecules (collagen, fibronectin, etc.), soluble mobilized macromolecules (e.g., growth factors, transcription factors, cytokines), and also from the transmembrane proteins of neighbouring cells [32]. Regarding bone matrix proteins, osteoblasts are responsible for the production of most of the organic components of the matrix, including collagenous and non-collagenous matrix components, which not only serve to structurally support cells but also to provide a variety of chemical cues that regulate functionality. Bone is around 30% organic, 90% of which is collagen type I. The remaining 10% are non-collagenous proteins and include proteoglycans, matrix proteins, growth factors, and cytokines. The most abundant protein in bone matrix is osteonectin, also known as SPARC (secreted protein, acidic and rich in cysteine), followed by osteocalcin (OC). Other extracellular glycoproteins as osteopontin (OPN) and thrombospondin (bone sialoproteins) are also present. OC and OPN are well-used biomarkers of bone formation. Bone matrix also contains proteoglycans (e.g., biglycan and decorin) that can influence cell behaviour [24, 25, 33-35]. Mobile biochemical cues directing cell behaviour can be produced by local osteoblasts or delivered via the blood stream. Transcriptional and growth factors such as platelet-derived growth factors

(PDGFs), bone morphogenetic proteins (BMPs), insulin-derived growth factors (IGFs), and transforming growth factor-β (TGF-β) have indisputable roles in the facilitation of osteoblast proliferation, differentiation, and subsequent bone formation and regulation [25, 26]. In addition to growth and transcriptional factors, systemic and local hormones such as parathyroid hormone influence osteoblast proliferation and differentiation [36].

In the event of an injury, bone regeneration is a complex, well-orchestrated physiological process of bone formation. The bone tissue injury initiates a cascade of events involving a number of cell types (i.e., neutrophils, macrophages, fibroblasts and MSCs) and intracellular and extracellular molecular-signalling pathways, with a definable temporal and spatial sequence, in an effort to optimise skeletal repair and restore skeletal functionality. Unlike other tissues, a number of bony injuries (fractures) self-heals without the formation of scar tissue, and bone is regenerated with its pre-existing properties largely restored, and with the newly formed bone being eventually indistinguishable from the adjacent uninjured bone [29, 37]. However, for a variety of reasons (such as bone defects size, infection, and many others), injured or diseased bone may not be capable of repairing itself. For such clinical scenarios, an appropriate biomaterial must be used to correct the bone defect.

1.2. Bone biomimetics biomaterials

Biomimetism and bioinspiration represent important tools for the design and the synthesis of innovative materials and devices. Biomimetism for bone applications can be conducted at different levels of physical and chemical properties of the synthetic materials and they should mimic all the characteristics of biological tissues, in order to optimize their interactions, and to mimic biogenic materials in their functionalities [22].

Up to now, a variety of synthetic materials have been investigated for bone applications including metals, ceramics, polymers, and composites of these. Obviously these materials differ widely in both chemical and mechanical characteristics; however, they all have properties that make them ideal for use in bone applications [3, 33, 38, 39].

Metals and metallic alloys as titanium and its alloys, stainless steel and cobalt-chromium among others, are known as being biocompatible, strong, easy to give shape and relatively inexpensive. They are frequently used, in dental and orthopaedic

applications, to replace and offer support for damaged and healing bone due to the high mechanical strength and fracture toughness. However, metals generally have a modulus higher than that of bone which may induce stress shielding and do not biodegrade, which requires additional surgery and may impede native tissue ingrowth. Additionally, there are concerns related to metal ion and/or particles release through corrosion or wear processes that cause inflammation and allergic reactions, affecting biocompatibility and tissue loss [40-42]. Considering the limited utility of generally non-degrading synthetic bone scaffolds or fixation devices, the only realistic options for bulk biomaterial selection are ceramics and polymers. Due to their biocompatibility, tunable degradability, processability, and general versatility, polymers, copolymers and polymer-ceramic composites are the principal materials investigated for the development of synthetic bone scaffolds [1]. The most commonly studied natural polymers for the purpose of bone tissue engineering are collagen/gelatin, chitosan, silk, alginate, hyaluronic acid and peptides [43-46]. The drawbacks of natural polymers include a higher risk of infection, fixed degradation rates, and immunogenicity [1]. As an alternative, there are the synthetic polymers as polyesters [3, 33].

Last but not the least, ceramics are clearly the most biocompatible functional materials. They are generally defined as ‘inorganic, non-metallic materials’ and three main families are well-known: calcium phosphates (CaP), glasses and glass ceramics. Commonly, bioceramics are classified as bioinert or bioactive. An inert material can be biocompatible, but elicit minimal biological response from the physiological environment; this is, for instance, the case of alumina and zirconia used in the production of femoral head implants. On the contrary, bioactive materials present a reactive surface which allows the formation of bone tissue on its surface [38, 47, 48]. Bioactive ceramics can be further categorized as resorbable or non-resorbable. Overall, ceramics are widely used as an implant material but present particular problems regarding their mechanical properties in terms of fracture toughness and fatigue resistance [49]. Among them, CaP is an obvious choice and has consistently shown excellent cellular and tissue responses *in vitro* and *in vivo* as an optimal material to aid bone repair, replacement and regeneration as they bond to bone and enhance bone tissue formation [17, 50, 51]. It has been widely used as bone substitutes in the form of powders, granules, dense and porous blocks, mouldable paste and various composites

[52]. There exists a family of CaP and the properties of each compound can be characterised according to the proportion of calcium to phosphorus ions in its structure. One of the most widely used synthetic CaP ceramics is HA due to its chemical similarities to the inorganic component of hard tissues which makes it widely used in both research and clinical fields [49, 53, 54].

1.2.1. Hydroxyapatite

Synthetic HA is a mineral from the family of apatites with chemical formula: $Ca_{10}(PO_4)_6(OH)_2$ and a hexagonal crystalline structure. It has a theoretical composition of 39.68 wt% Ca, 18.45 wt% P; Ca/P wt ratio of 2.151 and Ca/P molar ratio of 1.667. It has higher thermodynamic stability in aqueous media than other CaP ceramics within a pH range of 4.2-8.0 [49, 53, 54]. As aforementioned, the close chemical similarity to the inorganic component of natural bone matrix has led to extensive research efforts to use synthetic HA for a variety of biomedical applications [22]. As a bioactive ceramic, synthetic HA exhibits strong affinity to host hard tissues and the chemical bonding with the host tissue offers a greater advantage compared to most other bone substitutes such as allografts or metallic implants [50].

Besides its inherent osteoconductive and osteoinductive capabilities, physicochemical properties of synthetic HA crystals can be tailored to optimize their specific biomedical applications. Moreover, in biological conditions, the crystal structure of HA can accommodate extensive substitution of ions, so that human bone is, which affect both its cationic and anionic sublattices, crystal morphology, crystallinity, solubility and thermal stability [20, 22, 55].

Nowadays, through nanotechnology, it is possible to synthesize inorganic crystals within the nanometric range that offer some specific improvements compared to micro-structured HA. The importance of synthetic nanosized particles relates to the natural HA crystals seen in biology. As previously mentioned, the *in vivo* HA crystals are nanoscopic. Accordingly, synthetic inorganic crystals with nanometric dimensions can improve the biological responses of HA as a result of high bioresorbility, actual surface area, and surface structural disorder which increase crystal bioreactivity [2, 22]. At present, it is well established that nanoscale architecture of the material itself affects

the amount, distribution, density, conformation and orientation of adsorbed proteins and thereafter, will influence osteogenic cells [56-59]. For instance, Webster *et al* [60] have shown the effect of nanoscale topography in promote increased selective vitronectin adsorption (a protein that mediates osteoblast adhesion) that further enhances osteoblast activities as differentiation and proliferation. In a more recent work, Ribeiro *et al* [61] found that nanohydroxyaptite (nanoHA) substrates had an important role in the adsorption behaviour of fibronectin as well as osteonectin, and clearly affected the MC3T3-E1 osteoblast morphology, distribution and metabolic activity. Overall, such physical feature may improve biomaterial osteoinduction and osteointegration [2].

Additionally nanoHA powders exhibit improved sinterability and enhanced densification (due to their greater surface area), which may improve fracture toughness, as well as other mechanical properties. Even so, its intrinsic hardness and lack of flexibility restrict its use mainly to non-load-bearing applications [2].

Common applications of synthetic nanoHA include bone augmentation, coating of metallic implants or acting as fillers in critical-sized defect in either orthopaedic or dental applications [21, 22, 62]. As artificial bone grafts, scaffolds of nanoHA closely simulating spongy bone morphology have been developed and have been used in clinical settings to increase bone regeneration in a variety of orthopaedic and maxillofacial procedures. The scaffold design must obey key requirements as very high porosity with full interconnectivity. Such porous architectures define the mechanical properties of the scaffold, as well as the initial void space that is available for regenerating cells to attach, migrate and proliferate in order to form new tissues (including new vascularisation) and the pathways for oxygen and nutrient supply and waste removal. The rate of scaffold degradation must be tuned so that it provides the necessary structural support until the newly grown bone has sufficient mechanical strength to replace this supporting activity. At the end, the scaffold should be able to modulate osteogenesis and promote bone ingrowth, leading to successful tissue regeneration [33, 63-69]. As a coating material for implants, nanoHA stimulates bone growth around the implant creating strong bone-HA-prosthesis bonds and therefore, promoting direct osteointegration with juxtaposed bone. The presence of HA coatings also prevents the formation of fibrous tissue that could occur due to micro-movements of an uncoated titanium implant and also aid to meet the aesthetic requirements of some

implants [70-72]. Clinical results for HA-coated implants reveal that they have much longer life times after implantation than uncoated devices and they have been found to be particularly beneficial for younger patients [49].

Additional applicability has been found for nanoHA particles including the design of controlled release systems. HA is known for its binding capability to a wide variety of molecules either pharmaceutical species (e.g., antibiotics, anti-inflammatory drugs, and anticancer and anti-metastatic drugs) or nucleic acids for gene therapy. NanoHA solubility *in vivo* and its efficient cellular uptake are contributing factors [1, 20, 73-76]. A recent interesting method to use nanoHA has been to couple it with magnetic particles or to polarise it [21].

2. Implant-related infections

Bone-related implants should function in an appropriate fashion both mechanically and biologically, however, the surfaces of commonly used orthopaedic biomaterials are susceptible to colonization by microorganisms [77, 78]. Such colonization not only does precede acute and chronic infections, but it may also adversely compromise the functionality and performance of the implant itself [79, 80]. Therefore, the occurrence of infection seriously impairs the healing and regenerative capacity of a tissue and remains a major limitation in the long-term utility of orthopaedic implants leading to implant removal and consequent increased morbidity and even mortality [81].

While international efforts to minimize the risk of these infections are underway [82], orthopaedic implant-related infections or bone-associated infections continue to occur in large numbers. Current estimates suggest that the occurrence of infection after internal fixation varies between 0.4% and up to 16.1% depending on the type of fracture. After arthroplasties, peri-prosthetic joint infections occur in 0.3-1.7%, in 0.5-2% and in 2-9% of patients after total replacement of the hip, knee and ankle, respectively [83]. In addition to human pain and suffering, direct medical costs associated with such infections are extremely high and often result in the removal of the orthopaedic implants and the need for a follow-up operation [81]. Revision cases with tissue debridement carry an increased risk of infection [84]. The incidence rate is therefore higher, with an estimated rate of 3.2 to 5.6% for both hips and knees [85]. Even if it failures concern a minor proportion of patients, the huge numbers of individuals nowadays bearing permanent implants imply that, at the end, the overall impact on the entire population and on the costs for the national health systems are enormous [86, 87]. Longitudinal studies in industrialized countries have indicated a clear trend for further increase [88]. The overall infection burden is projected to raise by 4% between 2005 and 2030 for both primary and revision hip and knee arthroplasties. Higher life expectancy, advances in medical technology and treatment of younger patients will lead to greater demand worldwide for medical implants and eventually an increase in implant-associated infections that poses a serious threat for modern medicine [89-91].

The classification of implant-related infection considers either the type of pathogenesis or the onset of symptoms after implantation. Pathogenetically, inoculation occurs either exogenously or haematogenously [92]. Exogenous infections typically take place during surgery or immediately thereafter and most likely occur by inoculation with only a few microorganisms from the ambient atmosphere of the operating room, surgical equipment and clothing worn by medical professionals or resident bacteria on the patient's skin or mucous membranes. On the other hand, haematogenous infections are acquired via bloodstream by bacteria already in the body at any time after surgery [93, 94]. Even though, the risk has been reported to be highest within the first year after implantation and this might be related to the presence of a foreign body which impairs the local host defence [95-97]. The interstitial milieu surrounding prosthetic implants is known to represent a region of local immune depression and a *locus minoris resistentiae*, often referred to as immune-incompetent fibro-inflammatory zone [98]. This immune deficiency leads to a reduced ability to clear microorganisms from the vicinity of the biomaterial, and any contaminating microorganism are therefore more likely to cause a implant-related infection [99]. In fact, experimental models have well enlightened that the critical dose of contaminating microorganisms required to produce infection is much lower when a foreign material is present at the surgical site [80].

Often, implant-related infection is classified according to the time of manifestation after implantation. Considering the novel classification proposed by Zimmerli, early infection occurs within the first 3 weeks postoperatively and is typically caused by highly virulent microorganisms such as *Staphylococcus aureus* or *Escherichia coli*. Delayed infection manifests between 3 and 10 weeks postoperatively and, in most of these cases, microorganisms of low virulence such as coagulase-negative staphylococci (CoNS) or *Propionibacterium acnes* are responsible. Finally, late infection occurs more than 10 weeks after implantation. Both early and delayed infections are usually exogenously acquired while late infections typically occur after a symptom-free postoperative period and are caused by haematogenous seeding or by recurrence of inadequately treated early infection [83].

Prophylactic administration of antibiotics has been done routinely to patients who receive an orthopaedic device in order to prevent perioperative infection [100]. For optimal efficacy of the prophylactic agent, antimicrobial inhibitory concentrations must

be achieved in tissue at the time of incision and last during the entire procedure. Additionally, the literature strongly suggests that prevention or eradication of such infections is better achieved by using combinations of antibiotics rather than single therapy [85, 95, 101]. Overall, prophylactic administration has been shown to reduce the relative risk of wound infection by 81% compared with no prophylaxis [102]. The conventional systemic administration of antibiotics has however some major drawbacks including systemic toxicity with associated renal and liver complications, poor penetration into ischemic and necrotic tissue typical of post-traumatic and post-operative tissue, and the need for hospitalization [103]. Moreover, prolonged use of antibiotics at higher doses to reduce the prevalence of such infections may lead to drug resistance, and potentially compromise bone growth, immune system surveillance and implant osteointegration.

Other important phenomenon in the pathogenesis of implant-related infection is the survival of bacteria in the tissue surrounding implants. Though macrophages and granulocytes are present around an implant, the microorganisms cannot be cleared, due to the frustrated phagocytosis caused by the implantation of a biomaterial [96, 97]. In living bone tissue excised from a patient with recurrent, long-term osteomyelitis, bacteria were observed inside osteoblasts and osteoclasts, even in high numbers within resident macrophages [104-106]. The persistence of intracellular pathogens around an implanted biomaterial is therefore a major concern, as these bacteria are not as susceptible to antibiotic treatment as those associated directly with the implant which may represent a focus for clinical relapse episodes [107].

Implant-related infections are not only a consequence of host factors (such as obesity, rheumatoid arthritis, diabetes mellitus and immune-compromised status) [108]. The infecting agents as well as the characteristics of the implanted device including size, shape, material, topography and intended use are important variables [78, 109] (Figure 2). Of these three, microbial factors are probably the most important in the pathogenesis of implant-associated infection, whereas implant features are the most amenable to modification with the objective of preventing infection.

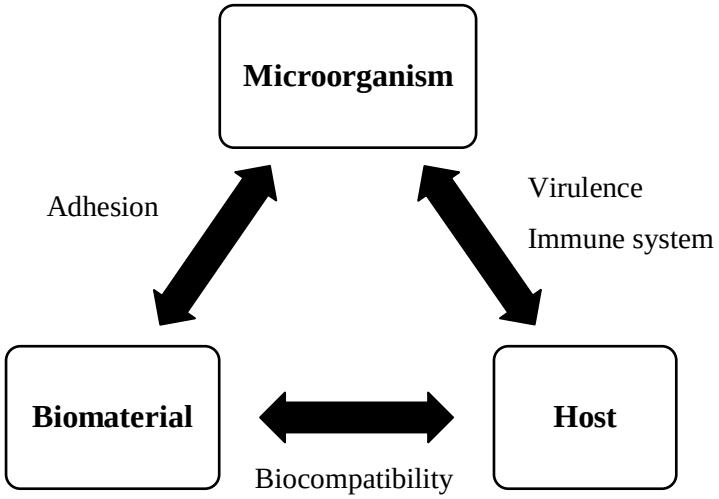

Figure 2. Interaction between the microorganism, the biomaterial and the host in the pathogenesis of implant-associated infections (adapted from [95]).

As mentioned earlier, the orthopaedic implants must, among other features, be made of non-cytotoxic materials and obtain mechanical stability with the adjacent bone and soft tissue. Concerning integration of orthopaedic implants, Gristina coined the phrase the 'race for the surface' suggesting that host cells and bacteria compete for positions on the implant's surface [110]. A 6-h post implantation 'decisive period' has been identified during which prevention of bacterial adhesion is critical for the long-term success of an implant. Over this period, an implant is particularly susceptible to surface colonization and at extended periods, certain species of adhered bacteria are capable of forming a biofilm at the implant-tissue interface which is considered the primary cause of implant-associated infection. Implantation will be therefore successful only if tissue integration occurs prior to considerable bacterial adhesion [110, 111].

Accordingly, orthopaedic devices are expected to stimulate host tissue integration and prevent microbial adhesion and colonization. However, the balance between these two requirements is often challenging. Biomaterial surfaces that facilitate host cell adhesion, spreading and growth are also favourable to microorganisms that share many of the same adhesive mechanisms as host cells. For instance, several bacteria have adhesins for fibronectin, which is a host protein that is frequently

associated with bacterial attachment to surfaces, followed by fibrinogen/fibrin, collagen, laminin and vitronectin [112]. On the other hand, surfaces and coatings designed to prevent bacterial colonization and biofilm formation may not effectively integrate with host tissues. Thus, the challenge is to develop new infection-resistant surfaces without further impairing, the local host immune competence or the potential for tissue integration [113].

2.1. Biofilm formation

Upon adhesion to a surface (inanimate material or tissue), replicating adherent unicellular individuals (bacteria or fungi) can arrange themselves into a complex tertiary structure displaying spatial and functional heterogeneity, encased in an ECM within which they are protected from a wide variety of antimicrobial factors [112].

This highly protective biofilm phenotype enables microorganisms colonizing an implant surface to evade antibiotics and host immune responses. Microorganisms growing as biofilms are significantly less susceptible to antibiotics therapy and host immune defences than are free-floating (planktonic) forms of the same microorganism [114, 115]. It has been found that killing bacteria in a biofilm sometimes requires approximately 1000 times the antibiotic dose necessary to achieve the same results in a suspension of cells [115-118]. Therefore, systemic antibiotics are able to eliminate planktonic organisms but are often ineffective in treating infections resulting from biofilm-embedded organisms. Thus, while the planktonic growth mode is important for the bacterial spread, biofilms are necessary to allow bacteria to persist and to resist adverse environmental conditions that may lead to persistence of infection, despite continued aggressive antibiotic treatment, for up to several years before awakening in more virulent modes [119].

Microbial adhesion to an implant surface is a dynamic process and, conceptually, can be divided into two essential phases: reversible and irreversible. The former is mechanically and biologically less stable than the latter, and it involves reversible cellular association with the surface over the first 1-2 hours post-implantation and it depends on the cell surface characteristics of the microorganism and on the nature of the biomaterial. This non-specific association is mediated through long (e.g.,

gravitational, van der Waals, and electrostatic interactions) and short (e.g., hydrogen bonding, dipole-dipole, ionic, and hydrophobic interactions) range forces. The second and irreversible phase begins approximately 2-3 hours later and is characterized by stronger adhesion between the bacteria and the biomaterial (Figure 3). Specific chemical reactions between compounds on the cell and substrate surfaces result in irreversible molecular bridging [111, 120, 121].

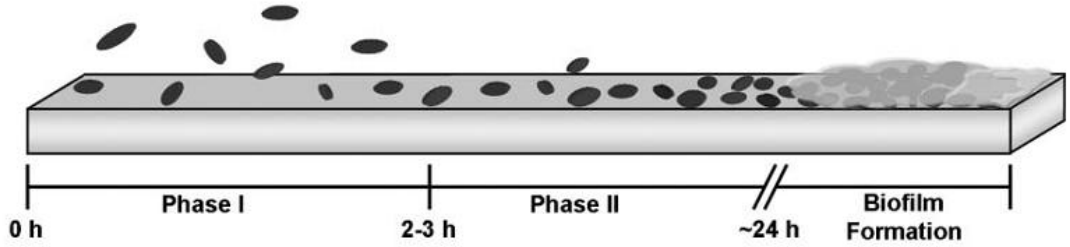

Figure 3. Representation of microbial adhesion to a biomaterial substrate. In phase I, the adhesion of planktonic microorganisms involves reversible cellular association with the surface. During Phase II, bacteria undergo irreversible molecular bridging with the substrate through cell surface adhesin compounds. After approximately 1 day, biofilm maturation and growth of the three dimensional community occur, surrounded by a self-produced ECM, that protects the sessile population from host defences and systemically-administered antibiotics (Adapted from [111]).

Regarding to the entire process of biofilm formation *in vivo*, the body typically reacts to biomedical implants by rapidly coating them with a conditioning film consisting of host plasma and connective tissue proteins and glycoproteins, such as fibronectin, vitronectin, fibrinogen, albumin, laminin, immunoglobulins and some plasma/tissue lipids. The exact format of the conditioning film is dependent on implant surface chemistry (charge and hydrophobicity), topography, the anatomic site and exposure time. This conditioning film affects then all the post-operative downstream events. Many of those proteins subsequently serve as specific receptors for colonising microorganisms or incoming mammalian cells. Regardless of what material is employed (e.g., metal, ceramic, polymer), the surface chemistry is instantly altered by this macromolecule adsorption [122-125]. Biofilm formation continues with the transport of planktonic cells to the substratum, which is governed by a combination of transport mechanisms (i.e., diffusion, convection, sedimentation, and motility) [120]. Once at a

substratum surface, microbial cells adhere, as previously described, by non-specific and thereafter, specific receptors [120, 121, 126]. Multiple specific receptors on the cell surface, called adhesins, bind to host molecules and different microorganisms use different adhesins to colonize implant's surface [79, 112]. After attachment, adherent (sessile) cells multiply and accumulate in multi-layered cell clusters which require intercellular adhesion, culminating in the formation of microcolonies. These organized structures are then surrounded by a self-produced ECM. Additionally, sessile cells up-regulate the secretion of certain cell signal molecules that orchestrate community-wide phenotypic responses, through a process termed quorum-sensing (QS). Thus, bacteria can act as a population instead of as individuals [127-130]. Biofilms continue to mature by consuming soluble nutrients and recruiting other microbial species or mammalian cells (e.g., platelets) [131]. At this stage, biofilm morphology and topography becomes very distinct with mushroom-like macrocolonies [132]. The formation of cavities or hollow channels all over the biofilm is evident and they provide to the biofilm the transport system necessary to guide water, nutrients, planktonic bacteria and waste disposal throughout the community. Depending on other quorum cell signals, focal areas of the biofilm dissolve and the liberated microbial cells can be carried downstream giving rise to septicaemia or spread to another location where new biofilms can be formed [133].

In most biofilms, the microorganisms account for less than 10% of the dry mass, whereas the matrix can account for over 90% and is one of the most notable characteristic of adherent cells colonizing medical implants. Water is by far the largest component of the matrix, but the extracellular material is also composed by a variety of biomolecules known as extracellular polymeric substances (EPS), frequently referred to as glycocalyx or 'slime', which are originated from both the microorganisms and the surrounding environment. The EPS are mainly polysaccharides, proteins, nucleic acids and lipids; that forms the basis of the three-dimensional structure of the biofilm and immobilize the cells, keeping them in close proximity to each other [112, 131]. This physical proximity enables horizontal genetic transfer; in fact, the conjugation frequency appears to be higher in bacteria growing in the sessile mode than in the planktonic mode. As a result, microbial biofilms provide a suitable environment to facilitate the cross-breeding of resistance genes [134]. Additionally, the mutation rate of

biofilm-growing organisms is significantly increased compared with planktonically growing isogenic ones [135].

The biofilm resistance is multifactorial and may vary from one organism to another. This combination of factors makes biofilm eradication difficult [117]. Overall, three mechanisms have been proposed to explain the general resistance of biofilms to antimicrobial agents. The first is the barrier properties of the ECM. This mechanism might be more relevant for reactive, charged or large antimicrobial agents that are neutralized or bound by the EPS and are effectively 'diluted' to sub-lethal concentrations before they can reach all of the individual microbial cells within the biofilm. The hydrated matrix might also protect against antibody opsonisation and phagocytosis, UV light, dehydration, and might localize antibiotic-degrading enzymes [131, 136]. Even though, not all antimicrobial agents are equally affected; glycopeptides such as vancomycin and teicoplanin were significantly affected, whereas agents such as rifampin, clindamycin, and the macrolides were either unaffected or minimally affected [115, 137]. Although the presence of the matrix undoubtedly retards the diffusion of antimicrobial agents, the poor penetration does not fully account for the observed biofilm drug resistance.

The second protective mechanism could involve the physiological state of biofilm-growing organisms. Biofilms are composed by a heterogeneous population of microbial cells that exhibit different metabolic properties creating multiple microniches [138]. Cells that are embedded deep within the biofilm might grow at a slower rate due to the lack of nutrients and oxygen in comparison with those located near the surface of the community. The formation of these starved, stationary phase dormant zones in biofilms seems to be a significant factor in the increase insensitivity to antimicrobial compounds, particularly against antibiotics such as β-lactams, which are effective against rapidly dividing Gram-positive bacteria. However, arguably all antibiotics require at least some degree of cellular activity to be effective, because the mechanism of action of most antibiotics involves disruption of a microbial process. Therefore, pockets of cells in a biofilm in stationary phase dormancy might represent a general mechanism of antibiotic resistance [112, 139, 140].

A third mechanism of protection could be the existence of sub-populations of resistant phenotypes in the biofilm that have been referred to as 'persisters'. These

comprise a small fraction of the entire biofilm population and are in a particular physiological state with low levels of translation but a unique gene expression profile being inherently resistant to the actions of antimicrobial agents [141-143].

Accordingly, strategies to further reduce the rate of implant-related infections must target specific mechanisms involved in biofilm formation namely inhibition of microbial adhesion to the surface (first line of defence), interference with the signal molecules modulating biofilm development (second line of defence) or the possibility of disrupting biofilm matrix (third line of defence).

2.2. Etiologic agents of implant-related infections

The aetiology of implant-related infections is generally characterised by a broad variety of Gram-positive and Gram-negative bacteria as well as yeasts. Still, a high prevalence of staphylococcal species is listed (approximately 75%), first of all the two biofilm-forming species *S. aureus* and *Staphylococcus epidermidis* [87, 144, 145]. The former is a pathogen colonising the nostrils of a large proportion of the human population and the principal aetiological agent of nosocomial infections. Implant-related infections caused by *S. aureus* account approximately 35% of all the isolates, proceed rapidly and are generally more severe than *S. epidermidis* infections, followed by serious complications as severe sepsis, septic thrombosis and/or several deep-seated infections (endocarditis, osteomyelitis and other metastatic infections) [87, 146, 147]. On the other hand, *S. epidermidis* is a common saprophytic bacterium inhabitant of the human skin and mucous membranes, which has progressively emerged as a main opportunistic species with the growing use of implant materials and represents roughly 30% of all the isolates [87, 148]. Apart from these two leading species, other staphylococci are emerging as new pathogens causative of implant-related infections. Among them other CoNS as *Staphylococcus haemolyticus* and *Staphylococcus warneri* contributing to an additional 10% of the infections. These bacteria take advantage of the weakening of the body defences at the implant surface-tissue interface since outside the setting of a medical device they rarely cause infections [80, 87, 94, 144, 149, 150]. Also *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and bacteria of the *Enterobacteriaceae* family are commonly isolated from infected orthopaedic implants

[87, 144, 145]. Conversely, microorganisms of the *Propionibacterium* genus, in particular *P. acnes*, is commonly recovered from orthopaedic implants albeit it is a commensal species from normal skin flora and often considered non-pathogenic [151]. With regard to yeasts, the percentage of infections related to orthopaedic joint prostheses caused by *Candida* ssp is less than 1%, considering a rate of infections of 1 to 3% this means 1-3 events per 10^4 patients [152]. *C. albicans* represents the predominant and most virulent species among all isolates however, the importance of infections caused by *Candida* non-*albicans*, and other unusual yeasts (e.g., *Malassezia* spp, *Rhodotorula* spp, *Hansenula anomala*) has emerged over the last decade [153-156]. Polymicrobial infections are observed in about 10-11%, and no organism is isolated in 10-30%, depending on the quality of the diagnostic procedure and preceding antimicrobial therapy [144, 157], meaning that, overall, these numbers may be underestimated and most probably are also increasing. An additional concern in recent years has been the increased number of reported infections due to antibiotic-resistant bacteria. In a large surveillance on surgical site infection after orthopaedic interventions, 59% of the isolates were methicillin-resistant *S. aureus* (MRSA) [91, 144, 158].

3. Biomaterial modifications to mitigate infection

Despite considerable recent progress in the development of nanobiotechnology and nanofabrication techniques, the quest to design and fabricate new antimicrobial surfaces as an integral component of advanced biomaterials remains a high research priority. The requirements that antimicrobial biomaterials need to cover are very broad, mostly depending on the type of biomaterial application [113, 159].

Antimicrobial surfaces can be categorized as passive or active depending on whether or not there are antibacterial agents delivered locally. Passive surfaces do not release bactericidal agents to the surrounding tissues; they only act on local bacteria that contact the surface. These surfaces may repel or resist the initial attachment of bacteria by either exhibiting an antibiofouling effect or by inactivating any cells coming into contact with the surface exhibiting a bactericidal effect. Antibiofouling surfaces impair cellular attachment due to the presence of an unfavourable surface chemistry and/or topography so that conditioning film do not form and/or bacteria-substrate interactions are not favourable; while bactericidal surfaces disrupt the cell upon contact, causing cell death [160-163]. On the other hand, active surfaces are designed to temporarily release high fluxes of pre-incorporated agents immediately following the implantation to down-regulate infection. Those agents may have a direct antibacterial activity (e.g., antibiotics, silver, and nitric oxide), as well as an indirect antibacterial effects (e.g., antibodies) [103, 111]. Passive surfaces are preferred as long as their antibacterial ability is strong enough to prevent bacterial adhesion. However, the effectiveness of passive coatings is limited and varies greatly depending on the bacterial species and can also potentially be masked and inactivated when filmed by the host proteins present in protein-rich physiologic fluids. The efficacy of active coatings is directly related to the amount of the antimicrobial substance released that is influenced by the processing parameters, loading dose, applied technique, molecular size of the drug and the implants' physicochemical properties [111, 164]. Antibacterial surfaces can be further categorized according to their functionality as mono- or multi-functional. The latter are expected to simultaneously target multiple biological tasks [163].

The following sections reviews some of the most successful approaches for the prevention of implant-associated infections, as well as promising perspectives for the

development of novel devices refractory to microbial adhesion, colonization and biofilm formation.

3.1. Antibiofouling surfaces

Modifications of the chemistry or the micro/nanotopography of the out-layer of an implant are key approaches in the development of innovative, adhesion-resistant materials or even microbial repellent surfaces since they play crucial roles in the kinetics of microbial adhesion. Alterations on such physicochemical properties are a relatively simple and economic way to counteract bacterial adhesion [113, 145, 165]. An efficient anti-adhesive surface should not only limit bacterial but also host proteins interaction to surfaces, therefore avoiding the formation of a conditioning film which may favour bacterial adhesion. In most cases, the implants are in contact with protein-rich solutions and, as a result, rapidly formed conditioning film can be, in fact, the real interface with bacteria [166, 167].

Anti-adhesive surfaces in protein-low and protein-free media are usually inert material surfaces. They can be obtained acting on the biomaterial surface chemistry or on the surface morphology/topography. The same types of inert surfaces often exert their antifouling activity even under protein-rich fluids, as they scarcely bind 'host adhesins'. In protein-rich fluids the dynamic of bacterial adhesion acquires a further degree of complexity. Under these circumstances, adequate surface chemistries may impair the adsorption of host adhesins, thus hindering the interactions with bacterial proteins. Therefore, conditioning protein-surfaces and/or protein-bacteria interactions are good strategies to inhibit bacterial adhesion to a specific biomaterial. The adsorption of proteins on a surface can be reduced either by altering the interaction potential (so that protein-surface interactions are suppressed) or by slowing down the rate of adsorption through high potential barriers for the interaction [145].

Regarding biomaterial surface hydrophobicity, it is generally accepted that hydrophobic bacterial cell surfaces adhere better to hydrophobic biomaterial surfaces due to a reduction in free surface energy [120, 126, 168]. Non-specific inhibition of adhesion is, therefore, obtained by coating the implant surface with hydrophilic materials. For instance, Patel *et al* [169] found that polyethylene oxide (PEO), a

hydrophilic polymer, significantly inhibited *S. epidermidis* biofilm formation over 48 hours. In another study, surfaces of metals were coated with polyethylene glycol (PEG) and PEO, and the authors concluded that such modifications were able to hamper protein absorption and subsequent bacterial adhesion to biomaterial surfaces since these are highly hydrated polymer chains [170]. In a more complex approach, effective low adhesion surfaces may consist in hydrophilic, highly hydrated and non-charged surfaces as those obtained with long chain polymers to form a brush-like structure on the surface. The density of the chains provides a steric barrier that repels bacteria by minimizing covalent interactions [171-173]. In an exhaustive study, Hook *et al* [174] assessed hundreds of polymeric materials using a high throughput microarray assay and identified a group of structurally related materials that substantially reduced the attachment of *P. aeruginosa*, *S. aureus* and *E. coli*. Then, coating silicone with these 'hit' materials showed to be effective at reducing *S. aureus* attachment *in vivo* in a mouse implant infection model.

Following an opposed trend, recently low-adhesive, super-hydrophobic and self-cleaning surfaces found in nature have been investigated for their potentially antibiofouling characteristics. Animals and plants have indeed developed fascinating strategies over millions of years to prevent efficiently the colonization of their surfaces by pathogens. For instance, the surfaces of insect wings, shark skin, and lotus leaves exhibit antibiofouling properties by preventing contaminating particles, algal spores, and bacterial cells from attaching to their surface [175-177]. Based on these evidences, super-hydrophobic microstructure arrays named as slippery liquid-infused porous surfaces (SLIPS) were produced on a silicon wafer and had proved to prevent bacterial attachment under flow conditions [178].

Besides chemistry, the morphology of a surface can be structured and directed to reduce adhesiveness to bacteria, even to achieve an enhanced bactericidal activity [179-181]. Recent studies with different nano-structured surfaces have demonstrated that surface topography can determine a different bacterial behaviour not just in terms of adhesion, but also in terms of cell metabolism [182-184]. Techniques such as micropatterning of antifouling surfaces have shown that bacterial-repellent and tissue-friendly surfaces may be achieved. For example, Wang *et al* [185] created surfaces with

submicrometer-sized, non-adhesive microgels patterned on an otherwise cell-adhesive surface.

3.2. Surfaces with anti-infective agents

Antimicrobial compounds can be incorporated in the bulk or in the coating of a biomaterial, based on their chemical compatibility, to inactivate any microbial cell around the implant. They can simply be mixed to the ingredients during the phase of production (e.g., in setting cements), *a posteriori* absorbed in permeable or porous biomaterials, covalently bound to functionalised coatings, incorporated in self-assembling mono/multilayer organic coatings, among others. The release can consequently occur by different modalities: diffusion to the aqueous phase, erosion/degradation of resorbable loaded matrices or hydrolysis of covalent bonds. Systems with different kinetics of delivery of the active principle have been achieved depending on the stability of the molecular bonds or of the rate of biodegradation/bioerosion of the matrices entrapping the antimicrobial agent [145, 186].

Numerous substances are known to possess antimicrobial properties yet such effect is rarely highly specific and uniquely oriented towards prokaryotic cells. Often it is associated to a certain degree of cytotoxicity, immunoreactivity, and genotoxicity which can potentially affect host cell viability in peri-prosthetic tissues and, in the presence of specific tissue tropisms of the chemical species released, even cells residing at distant anatomic sites [145, 163].

3.2.1. Inorganic agents

Silver has been described as one of the earliest materials to be intentionally used in biomedical applications for its oligodynamic antibacterial activity, i.e., exhibiting bactericidal/bacteriostatic activity at very low concentration, against a wide variety of Gram-positive and Gram-negative bacteria [187]. However, since its early identification as a convenient anti-infective biomaterial, the use of silver as bulk material in medical devices has progressively been ceasing over time. Following an opposite trend, the utilization of this element in thin nanocoatings, in doped solid or hydrogel materials, in

the formulation of bioactive alloys and glasses, and in form of micro- and nanoparticles has progressively been flourishing [188-192]. Also, some metal oxides (i.e., TiO_2, ZnO, SiO_2), have been explored due to their intrinsic antimicrobial properties on a wide spectrum of bacterial species [193-198]. Several studies had confirmed the improved antimicrobial effect of these metals as nanoparticles [188, 191, 194, 199, 200]. The nanoparticles can be either deposited directly on the surface of the implant or applied in a biomaterial matrix [201, 202]. For example, Alt *et al* [203] found that bone cement loaded with silver nanoparticles showed high effectiveness against multi-resistant bacteria in the absence of *in vitro* cytotoxicity. In a more recent study, a silver-coated megaprosthesis was able to reduce the infection rate in bone sarcoma patients from 17.6% (pure titanium) to 5.9% (silver group). Whereas 38.5% of patients in the titanium group ultimately had to undergo amputation when peri-prosthetic infection developed, these mutilating surgical procedures were not necessary in the study group [204]. Regarding copper, for instance, Cu-sputtered polyester has shown to have a very high bactericidal efficiency against a number of MRSA strains [205]. Similarly, nanostructured ZnO and TiO_2 were able to reduce *S. epidermidis* adhesion and increase osteoblast activity required to promote the efficacy of orthopaedic implants [206].

The antimicrobial activity of the majority of metals is closely linked to the ionic or nano form rather than to the bulk material. Dissolved ions are biochemically active agents that can interfere with critical enzymes of the respiratory chain, cell membrane permeability, hydroxyl radical formation, and subsequent DNA damage [197, 207].

Though metals show attractive characteristics as antimicrobial agents, further information is needed regarding both their stability in physiological fluids and their biological safety. Some studies have shown that such metals can adversely affect surrounding cells and lead to potentially harmful accumulation in distant locations, especially when in the form of nanoparticles [208, 209]. Additionally, there is still concern over the potential acquisition of resistance by bacteria to metallic compounds, especially with respect to silver. It is believed that silver resistance is considered widespread, although this not being confirmed by exhaustive tests and it overuse could potentiate the problem [210, 211].

3.2.2. Organic agents

Since its conceptualization by Buchholz and Engelbrecht in 1970, the use of antibiotic-loaded acrylic cement for the management of prosthetic joint infection has been common practice among many orthopaedic surgeons [212, 213]. Even now, antibiotic-loaded cements are frequently applied in orthopaedics, especially in association with the replacement of infected prostheses due to the high risk of relapse. Overall, antibiotic-loaded implant coatings present a straightforward approach for the prevention of implant-associated infections and have generally shown favourable results regarding their efficacy. The primary advantage of delivering antibiotics directly at the site of implantation is that high local doses can be administered without exceeding the systemic toxicity level of the drug. Therefore, enhanced efficacy can be achieved at the implant site. Localized administration also allows for the tailored selection of antibiotics toward specific pathogens associated with implant infections, circumventing potentially harmful side reactions in other parts of the body [111, 214].

The ideal antibiotic delivery system should release the drug at optimal bactericidal levels for an appropriate therapeutic time frame to prevent potential infection, followed by a sustained release at an effective level to inhibit the occurrence of latent infection [1, 103]. In the case of implant-related infections it is important to fight bacteria that were introduced during the implantation and also those introduced systemically afterwards. A sustained release (second phase) is therefore necessary. Nevertheless, it should be ensured that in the second phase, the antibiotic concentration is at an effective level. Studies have reported that sub-inhibitory concentrations of certain antibiotics can actually enhance biofilm formation and for sure they can act by selecting and promoting the emergence of new antibiotic resistant strains [215-217]. Any undesired effect of antibiotics on tissue integration of the implant should be minimized when designing the antibiotic delivery system.

Several antibiotics have been widely used, as well as delivery systems. Specially, HA coating is widely applied and the use of a bioactive ceramic coating as antibiotic carriers offers the added value of providing the physicochemical environment and structural scaffold required for bone-implant integration [218]. *In vitro* release of antibiotics from HA-coated implants has been reported for a broad range of drugs. For instance, Alt *et al* [219] investigate the antimicrobial effect of two different gentamicin-

HA coatings for cementless prostheses in a rabbit infection model. After 28 days, both gentamicin-coating types showed significant reduction of infection rates compared to standard HA coating. Afterwards, still in an *in vivo* model, that author assessed the effects of gentamicin-HA coatings on new bone formation, implant integration and biocompatibility. Both gentamicin coatings revealed good biocompatibility and bone integration, which was not statistically different when compared with pure HA [220]. In this context, several other studies have been conducted [221-226].

The antibiotic-loading can be performed by surface-adsorbed antibiotics through immersion in antibiotic solutions. Although simple, this approach leads to rapid antibiotic release, excluding it as a method for preventing implant fouling over long periods. Other strategies include the embedding of antimicrobial substances in nanoceramics or surfaces coated with covalently linked antibiotics [218]. Antoci *et al* [227] observed that covalently bonded vancomycin to titanium alloy (Ti) pins prevented *S. aureus* colonization. Moreover, these authors confirmed that the attachment remained stable under a number of conditions, including exposure to fluid environments, press-fit insertion into bone, and saturating levels of *S. aureus*. Besides Antoci work, several other authors have shown that they have effectively engineered a stable bactericidal surface [228, 229].

The clinical effectiveness of the aforementioned systems is most likely limited to infections caused by microorganisms that are sensitive to the specific antibiotic that has been coupled. It is also critical to be aware that, as previously mentioned, virulent strains of certain pathogens do not just colonize implant surfaces, but they are also capable to internalise within cells of the connective tissue. This occurrence is especially important in revision surgeries. Under such circumstances it is crucial to make use of antibiotics able to enter the membranes of eukaryotic cells and therefore reach intracellularly hidden bacteria. Otherwise, bacteria will survive all treatments well protected in the intracellular reservoir, ready to restart the infection once the local concentration of antimicrobial agent in the interstitial milieu will have lost its efficacy [80, 163, 217].

With the increasing number of antibiotic-resistant microorganisms, antimicrobial peptides (AMPs) are an extremely interesting group of anti-infective agents and currently sought as the next generation of antibiotics. AMPs are natural and

evolutionary conserved components of the innate immune system of all multicellular organisms to protect them against invading microorganisms [230]. Eukaryotic AMPs are small (10-50 amino acids), cationic and contain both hydrophobic and hydrophilic parts [231]. With a broad-spectrum of activity, not only against bacteria but also against certain viruses and fungi, their microbicidal action can be rapid through a number of different ways. Available data indicates that they act predominantly by disrupting the integrity of cell membranes through a strong electrostatic interaction between AMPs cationic residues and the negatively charged bacterial membranes, leading to instability, pore formation, osmotic changes and bacterial lysis [232-236]. However, an increasing number of peptides have also been described as acting on intracellular targets in bacteria, inhibiting protein or cell-wall synthesis or interactions with deoxyribonucleic acid (DNA) or ribosomal ribonucleic acid (rRNA) [237]. Accordingly, AMPs may represent excellent coating agents on a range of medical devices. They are far less susceptible to the development of pathogen resistance compared to conventional antibiotics, exhibit rapid and broad-spectrum killing profiles, inclusive against antibiotic-resistant bacteria, and are effective at low concentrations [230, 238-241]. The challenge remains being the immobilization of peptide molecules while keeping their accessibility and durable activity towards the surrounding bacterial cells [242]. Kazemzadeh-Narbat *et al* [243] coated the surface of titanium substrate with a thin layer of micro-porous calcium phosphate loaded with Tet 123 (a highly potent broad-spectrum AMP) and found that the resulting coatings were able to kill both *S. aureus* and *P. aeruginosa* within 30 minutes of exposure and the coatings were absolutely non-toxic toward osteoblast-like cells. In this scope, several other works can be found on the literature [244-248]. Therapeutic applications of AMPs are currently unavailable. Potential local toxicity, allergy, susceptibility to proteases and pH changes, high cost of peptide production constitute the main limitations associated to the use of AMPs [241, 249, 250]. Nevertheless, AMPs are evoking increasing interest and are inspiring the production of new synthetic compounds in an attempt to overcome these drawbacks and optimise the bactericidal activity [235, 251, 252].

In the large group of natural polymers, chitosan (CS), derived from chitin, is noteworthy due to its intrinsic antibacterial and antifungal activities, albeit a weak bactericidal activity, which is usually enhanced at low pH. Different mechanisms for its

antibacterial activity have been proposed, such as electrostatic interaction, chelating and hydrophobic effect [145, 253]. CS-based biomaterials have other interesting characteristics such as biocompatibility, minimal foreign body reaction and the possibility to be moulded in various forms such as porous structures, which makes them suitable candidates for orthopaedic applications [254]. Over the last decades, with the intent to exalt its antibacterial properties, a broad family of CS derivatives has been developed aiming at antibacterial usage in biomedicine, either alone or in combination with other antimicrobial substances like antibiotics or AMPs [253, 255-257]. Among recent researches, quaternised chitosan-loaded bone cement was able to prevent biofilm formation of *Staphylococcus*, including antibiotic-resistant strains, on the surface of bone cement, and down-regulates expression of genes encoding essential enzymes for biofilm biosynthesis, as well as enzymes responsible for antibiotic-resistance [258].

An innovative approach goes through the enhancement of body's natural defence systems to fight pathogens, thereby enhancing resistance to infection. Therefore, immunomodulatory proteins as chemokines are interesting anti-infective agents. In the early stage of infection, macrophages constitute the primary line of innate immune defence against most pathogens. In order to attract macrophages to the site of infection, one possible strategy is to deliver essential chemo-attractant proteins in the peri-prosthetic site. Among all the macrophage-recruiting chemokines, monocyte chemo-attractant protein-1 (MCP-1) is the most important for monocyte/macrophage recruitment in infection and inflammation [259, 260]. Based on this knowledge, Li *et al* [261] developed MCP-1 and interleukin-12 p70 (IL-12 p70) nanocoatings on orthopaedic implants and determined their anti-infection effectiveness using an open fracture infection model. The authors found that local MCP-1 therapy reduced *S. aureus* infection and influenced white blood cell populations while local IL-12 p70 treatment had a more profound effect on preventing *S. aureus* infection. Ultimately, this study demonstrates that this type of approach has therapeutic relevance in circumstances of impaired fracture healing in which macrophage performance is suboptimal.

Still considering the immune system, nitric oxide (NO) is a potent antimicrobial molecule produced by macrophages, among others cells, as part of the natural immune response to infection [262]. NO is a strong oxidizing agent capable of targeting important structures within bacteria cells, including DNA and proteins, after diffusion

across their cell membranes. Oxidation of DNA by NO directly or indirectly by a multitude of reactive intermediates is capable of inducing irreparable damage by breaking the DNA strand [111]. NO reaction product peroxynitrite has also been implicated in cell membrane destruction by lipid peroxidation [263]. This effect may compromise cell attachment and thereby contribute to lower levels of bacterial adhesion. Therefore, the use of NO release as an antibacterial agent to reduce the infection around implants has been proposed. For example, Nablo *et al* [264] has examined the benefits of NO-releasing sol-gels as potential antibacterial coatings for orthopaedic applications, and they observed that the adhesion capabilities of *P. aeruginosa*, *S. aureus*, and *S. epidermidis* are diminished in the presence of a low-level, surface-localized NO flux at both ambient and physiological temperature. Later on, the same group confirmed the ability of NO-releasing xerogels to reduce the initial adhesion of *P. aeruginosa* under dynamic flow conditions. NO release was also able to kill adhered bacteria in a dose-dependent fashion over extended periods [265]. One step ahead, Charville and colleagues examined the efficacy of NO-release to prevent bacterial adhesion in the presence of an adhesion-promoting protein layer. The authors found that NO release from xerogel polymer surface reduced *S. aureus*, *S. epidermidis* and *E. coli* adhesion, even in the presence of pre-adsorbed fibrinogen [266]. Additional works based on the NO-releasing materials can be found in the literature [267-269]. Despite the exciting results, it is important to finely tune the beneficial and toxic effects of NO, which is active at concentrations as low as the picomolar and nanomolar produced by immune system [270].

The aforementioned approaches are a number of potential tools for biomedical engineers to develop the next generation of biomaterials aiming the prevention of initial microbial adhesion and, therefore, the subsequent implant-associated infection. Although, fighting against initial adhesion is not always sufficient to avoid biofilm development. The progress in the knowledge of the molecular mechanisms involved in the physiology of biofilm formation of different microorganisms has recently made available new opportunities to contrast the establishment of biofilms on biomaterial surfaces [112, 271-274]. Biofilm disruption could *per se* have limited efficacy in the prevention of infections, but the combination of anti-biofilm strategies with the delivery of conventional antimicrobials agents may have promising synergistic effects.

A wide range of substances that affect specific mechanisms involved in biofilm formation have been identified and can be either grafted on biomaterial surfaces or released by appropriate systems. These active substances include molecules with different action mechanisms: enzymes capable of selectively degrading biofilm EPS (e.g., dispersin B or deoxyribonuclease I) exposing sessile microbial cells to antibiotics as well as to the host immune defence; bactericidal molecules capable of killing even metabolically quiescent bacterial cells within biofilms (e.g., lysostaphin, certain AMPs); molecules interfering with the QS system and inducing biofilm dispersion (e.g., furanones); molecules down-regulating the expression of biofilm EPS (e.g., *N*-acetylcysteine) or anyway reducing biofilm metabolism (e.g., hamamelitannin). Due to the diverse mechanisms of biofilm regulation that characterise different microbial genera and species, these substances often do not exhibit a broad spectrum of activity. Nevertheless, some of them selectively target staphylococcal species known to represent prevalent aetiological agents of implant-related infections. In view of the selectivity of their action, these molecules are generally expected to have a low impact on host cells and tissues [86, 275, 276].

The clinical potential of some of these molecules has already been estimated. For instance, dispersin B (DspB) is able to dissolve the staphylococcal ECM [276]. Several studies have demonstrated the synergistic antimicrobial and anti-biofilm activity between DspB and a large number of agents including cefamandole nafate [277], sodium dodecyl sulphate [278] and triclosan [279]. Accordingly, further characterization of these polymer-DspB-antibiotic systems sounds promising. Another promising enzyme is lysostaphin (LS). LS is an endopeptidase that disrupts the pentaglycine cross-linking bridges in the cell walls of staphylococci, especially on *S. aureus*, making their action highly active against both actively growing and quiescent bacteria, even on antibiotic resistant strains. Wu *et al* [280] found that LS is effective against planktonic *S. aureus* within minutes, as well as against *S. epidermidis*, albeit at higher concentrations. Additionally, the authors demonstrated that LS disrupted both *S. aureus* and *S. epidermidis* biofilms *in vitro*. In another study, Shah *et al* [281] observed that LS-coated catheters prevented biomaterial colonization by several strains of *S. aureus*, and the activity was maintained for at least 4 days. Concerning LS biocompatibility, Rawson *et al* [282] found that LS was biocompatible within the

reported biofilm inhibitory concentration ranges and supported osteoblast differentiation.

The QS system comprises several molecules that can be potential clinical targets since QS is known to be crucial for survival of biofilm bacteria. It is a system mediated by small signaling molecules, called autoinducers. Different classes of autoinducers are involved in QS such as oligopeptides in Gram-positive bacteria and *N*-acyl homoserine lactones (AHLs) in Gram-negative bacteria. There is also a family of autoinducers named autoinducer-2 (AI-2) which are present in both Gram-negative and Gram-positive organisms [130, 283, 284]. Deletions or inhibitions of this system obviated the whole process of biofilm formation for bacteria. Potent inhibitors of Gram-negative QS are the halogenated furanone and related synthetic derivatives that interfere with the QS by fixing on the binding site of AHLs [285, 286]. Alternatively, Baveja *et al* physically adsorbed a furanone on polymer materials while Hume *et al* covalently bound furanones to polymers commonly used for medical devices, and both studies have showed promising results, *in vitro* and *in vivo*, about furanones potential as a coating for biomaterials to control infection also caused by *S. epidermidis* [287, 288]. Similarly, strong inhibitors of Gram-positive QS were identified specifically RNAIII-inhibiting peptide (RIP). Several works have assessed the *in vitro* and *in vivo* efficacy of RIP [289-291]. For instance, bone cement beads loaded with RIP were implanted in rats and were able to prevent MRSA infection [292]. Overall, QS inhibitors and antagonists represent a promising strategy to counteract microbial adaptation to the host environment and the establishment of infectious processes [127, 293]. This knowledge from biofilm microbiology will be available for use in orthopaedics, and a detachment signal that triggers the natural detachment of cells from pre-formed biofilms also may be available in the near future [294].

Similar to the use of enzymes, most of these biofilm inhibitors prevent biofilm formation and restrict bacteria to the planktonic mode-of-growth, in which they are susceptible to host defences and antibiotics. Thus, their inclusion in coatings for orthopaedic devices would early interfere with QS system but an additional agent with antibacterial activity would improve the effectiveness of such approachs. Furthermore, special attention should be given to approaches based on biofilm disruption due to the resulting entrance of bacteria or groups of bacteria release from the biofilm into

circulation. These bacteria may not belong to either the planktonic or the biofilm phase and consequently may have a different pattern of antimicrobial susceptibility. A very interesting work by El-Azizi *et al* [116] showed that bacteria in the disrupted biofilms were as resistant as those in the intact biofilms at the minimum inhibitory concentrations of the antibiotics. At higher concentrations, bacteria in the disrupted biofilms were significantly less resistant than those in the intact biofilms but still more resistant than the planktonic cells. Therefore, the difficulty in treating the infections related to medical devices may not only be due to lack of eradication of the cells in the biofilm phase, but also due to resistance of bacteria disrupted from the biofilm. Even a combinatory therapy may be carefully evaluated.

3.2.3. Alternative approaches

The potential applicability of alternative 'biologic weapons' to prevent implant-related infections has been explored. One of these approaches is the enormous group of natural products. Natural products have proven to be highly efficient for the treatment of infections and, not surprisingly, the variety of drugs based on natural products is enormous. There are drugs with broad and narrow spectra for oral, topical or parenteral administration and with activities against almost all known pathogens. Therefore, the search for antimicrobial agents able to inhibit bacterial biofilm formation can also be extended to natural substances [295, 296]. However, their complex composition may impair the straight clinical application. Further studies are still needed to have a clear understand of their mechanism of action, albeit the presence of numerous compounds in their composition suggests that their activity is probably not attributable to one specific mechanism but results from a combination of several mechanisms involving synergic effects [251].

Another 'biologic weapon' that has gained renewed interest with the increased prevalence of antibiotic resistance is bacteriophage therapy [297, 298]. Bacteriophages are viruses that specifically infect bacteria. In particular, lytic phages bind to a membrane receptor, introducing phage DNA into the cell. This DNA is replicated and translated by the host bacterium, leading to phage replication, progeny assembly, bacterial lysis, release of progeny, and phage propagation to surviving bacteria [299].

The use of phages dates back to the first decades of the 20th century however, nowadays has increasingly been explored even for the prevention of implant-related infections [300, 301]. Attempts have been made using either the lytic bacteriophages alone [302] or in combination with an antibiotic drug [303]. For instance, Yilmaz *et al* [304] performed an *in vivo* study to assess if bacteriophages would be effective against biofilm-forming bacteria and these authors got to the conclusion that the combination of bacteriophage treatment with an appropriate antibiotic regime helped to dissolve the biofilm of both MRSA and *P. aeruginosa*. Overall, bacteriophages are inherently non-toxic and have minimal impact on the normal healthy flora. They have good cell-penetrative ability, so can readily disrupt and lyse biofilm cells [300]. Nevertheless, the application of phages for infection prophylaxis presents some limitations and drawbacks such as: phages generally exhibit narrow spectrum of activity; pre-exposures of the immune system to the phages can cause virus inactivation; safety concerns have been expressed for the internal use of high titres of phages, which could expose the patient to health risks still not totally explored; the procedure for coating the implant surface has to preserve intact phage stability and infectivity; and ultimately, in nature phages are implicated in the horizontal spreading of virulence and antibiotic resistance genes among bacteria [305]. To overcome these drawbacks bioengineered phages are a possibility [306]. For instance, engineered phages able to express DspB were successfully tested against *E. coli* biofilms [307]. Through these approaches, phages can be used both to dissolve the biofilm matrix and to kill microbial cells within the biofilm.

In a different approach, but with the same target, vaccines against appropriately selected patterns of bacterial adhesins appear an interesting and potentially effective control strategy since they could prevent initial bacterial adhesion to biomaterials [308]. The critical problem for vaccine development is the identification of a relevant antigen that is present in the planktonic and biofilm state of most clinical strains [309]. To overcome this issue, adhesin-targeting vaccines could be also assembled with others, to target diverse virulence factors, such as biofilm antigens or surface proteins other than adhesins, and thus to potentiate and expand their efficacy [310]. In this context, Brady *et al* [311] were able to generate a multicomponent vaccine using biofilm-specific antigens. The results obtained indicate that when vaccination was coupled with vancomycin treatment in a biofilm model of chronic osteomyelitis in rabbits, clinical

and radiographic signs of infection significantly reduced by 67 and 82%, respectively, compared to infected animals that were either treated with vancomycin or left untreated. In contrast, vaccination alone resulted in a modest and non-significant decrease in clinical (34% reduction) and radiographic signs (9% reduction) of infection, compared to non-vaccinated animal groups untreated or treated with vancomycin. It is not unfair conclude that the complexities of the biofilm architecture, with multiple microbiological communities and with various sites within the communities that can express different proteins required for survival, makes the development of a effective anti-biofilm vaccine a considerable challenge.

3.3. Multifunctional surfaces

The development of surfaces with multiple functionalities, for example both antibiofouling and bactericide properties, is quite appealing. In this framework, Gao *et al* [312] developed a specially structured infection-resistant coating on implants based on covalently grafted hydrophilic polymer brushes conjugated with an optimized series of AMPs. The polymer brush tethered AMPs showed excellent broad spectrum antimicrobial activity, as well as biofilm resistance *in vitro*. The biofilm resistance of the coating was attributed to the combined effect of polymer structure and the presence of AMPs, and the effect was correlated to hydrophobic/hydrophilic character of the coatings. *In vivo* studies demonstrated that the coatings were able to protect from bacterial infection and, the AMPs conjugated polymer coatings, were non-toxic to mammalian cells. Another interesting example is the work of Fullenkamp *et al* [189]. They synthesized a water-soluble PEG polymer that contain reactive catechol moieties which were then oxidized with silver nitrate, leading to covalent cross-linking and hydrogel formation with simultaneous reduction of Ag (I). Hydrogels were found to inhibit bacterial growth, consistent with the well-known antibacterial properties of silver, while not significantly affecting mammalian cell viability. In addition, thin hydrogel films were found to resist bacterial attachment, consistent with the antibiofouling properties of PEG. Immunotherapy (i.e., clinical delivery of externally-derived antibodies) may also be a strategy to design multifunctional surfaces. Treatment with exogenously-supplied immunoglobulin G (IgG) has been shown to diminish the

severity of infections and reduce bacterial adhesion to model surfaces. IgG opsonisation (i.e., antibody recognition of and binding to bacterial cell-surface antigens) inhibits bacterial adhesion by blocking cell-surface attachment factors or mechanism of transport (e.g., flagella); or targets them for phagocytic destruction by immune system components including neutrophils, monocytes, and macrophages [313-317]. Rojas *et al* [318] work describes the release of IgG from hydrophilic polyurethane hydrogel coatings and they found that these coatings were effective in reducing the adhesion of *E. coli*, which indicate that pathogen colonization and virulence in implant sites and on biomaterial devices could benefit from these two-way approaches.

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CHAPTER II

In vitro analysis of the antibacterial effect of nanohydroxyapatite-ZnO composites

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ABSTRACT

Hydroxyapatite (HA) is a biocompatible and bioactive synthetic material for biomedical applications as it binds to bone and enhances bone tissue formation. Particularly, nanophased HA can mimic the dimensions of constituent components of natural tissues; can modulate enhanced osteoblast adhesion and resorption with long-term functionality of tissue engineered implants. However, HA does not inhibit bacteria from adhering onto its surface, and this has implications in the bone healing process required for patient recovery, since infection can lead to the implant failure. In the present work a composite that combines the favorable biological characteristics of nanohydroxyapatite (nanoHA) and, simultaneously, possesses antimicrobial activity as expressed by ZnO was synthesized. To determine whether the size of ZnO particles was playing an important role in inhibiting bacterial growth, ZnO particle of different sizes (from the microscale down to the nanoscale) and concentration were incorporated into nanoHA and tested. The composite samples were characterized by SEM, FT-IR, XRD, XPS and zeta potential. The antibacterial activity of the composites was investigated, as well as the biofilm formation, using both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) microorganisms. The characterization revealed that ZnO particles were dispersed homogeneously within the nanoHA matrix. The composites antibacterial activity increased with decreasing ZnO particle size and increasing concentration. Biofilm formation tests revealed that the nanoHA-ZnO composites exhibit a strong effect against the common pathogens *S. aureus* and *E. coli*.

Keywords: nanohydroxyapatite, zinc oxide, composite, antibacterial, biofilm

1. INTRODUCTION

Hydroxyapatite is the most used calcium phosphate to be applied in implants due to its structural and chemical similarity with bone mineral component. It is highly stable in body fluids and is known for its exceptional biocompatibility, bioactivity, osteoinduction, and osseoconductivity properties [1], therefore it has been widely used clinically for coating metallic implants or filling small bone defects. Compared to conventional ceramic formulations, nanophased hydroxyapatite exhibits physicochemical characteristics similar to those of bone nanocrystals [2, 3]. The surfaces of these materials are generally designed to encourage tissue adherence, eventually leading to tissue or osseointegration but, unfortunately, this feature may also encourage bacterial adhesion. About half of the two million cases of nosocomial infections per year in the US are associated with indwelling devices [4]. Among surgical site infections, those related to implanted orthopaedic devices are of great relevance for public health due to the increasing number of aged and disabled patients requiring this type of surgical interventions [5]. Despite the low risk of infection, estimated to be in the range of 0.5%-5% for total joint replacements, they must be considered highly significant due to the serious consequences, which include prolonged hospitalization with systemic antibiotic therapy, several revision procedures, possible amputation, and even death [4, 6]. Such infections are difficult to treat with antibiotics because the bacteria causing the infection form biofilms on the implant surface. Once a biofilm has formed, the bacteria inside the biofilm are embedded in a polymeric matrix, protected from phagocytosis and antibiotics [7]. For this reason, a number of strategies for device-related infections prevention has been developed. Moreover, the increase in infections attributed to multi-drug-resistant *Staphylococcus aureus* has led to a growing interest in identifying and developing new strategies to reduce bacteria activity without the use of antibiotics [8]. The antibacterial agents can be classified into two categories according to their chemical composition: organic and inorganic. Organic antimicrobial agents are often less stable, particularly at high temperature or pressure, exhibit high decomposability and short life expectancy. On the other hand, inorganic antibacterial materials are robust and durable, and consequently have the key advantages of improved safety and stability [9-12]. As a consequence, inorganic materials such as metal and metal oxides have attracted attention due to their ability to resist harsh

process conditions. Among the metal oxides, zinc oxide (ZnO) has been shown to naturally reduce the activity of a wide range of bacterial strains, pathogenic and nonpathogenic, without the use of antibiotics [13, 14]. Several studies suggest that different morphologies (particle size and shape) and concentration of ZnO have different degrees of antibacterial activities [9, 10, 13, 15-17]. However, the mechanism of the antibacterial activity of ZnO is complex and still not completely understood, with different effects been suggested [8, 9, 11, 18-22]. The aim of the present research was to synthesize a composite that combines the favorable biological characteristics of nanohydroxyapatite (nanoHA) and, simultaneously, possesses antimicrobial activity as expressed by ZnO. Our attention was focused both on producing and characterizing nanoHA-ZnO composites and on evaluating the influence of ZnO particle size and amount on the bacterial activity and biofilm formation against a Gram-positive bacteria *Staphylococcus aureus* and a Gram-negative bacteria *Escherichia coli*, which are frequent etiologic agents on biofilm-related device-related infections [6, 23].

2. MATERIAL AND METHODS

2.1. Synthesis of nanoHA-ZnO composites

Zinc oxide powders were purchased from Sigma-Aldrich and used as received. The powders sizes given by the manufacturer were: particle size <1 μm, <100 nm and <50 nm. The composite samples were prepared by mixing ZnO powder with nanoHA (nanoXIM·HAp202; Fluidinona S.A., Portugal) at weight percentages (wt %) of 0, 2, 10 and 25%. The composite powders were subsequently pressed as cylindrical samples of 10 mm diameter in an uniaxial press (Mestra Snow, P3) under optimum pressure of 20 bar [24]. NanoHA ceramics are very fragile materials if not adequately heat-treated, as are most ceramic materials, therefore two different heat-treatments were applied based in a previous work [25], namely 830 and 1000ºC, with a 15-min plateau and applying a heating rate of 20ºC min^{-1}. The sintering cycle was completed with a natural cooling process inside the furnace (Commodore II, VPF, Jelenko).

2.2. Physicochemical characterization of nanoHA-ZnO composites

The samples surface morphology was analyzed using a scanning electron microscope (SEM; FEI Quanta 400 FEG/ESEM) under 2500x magnification, with 15 kV accelerating voltage. Carbon tape was used to fix samples to aluminum stubs and the samples were sputter-coated (SPI-Module) with a thin conductive film of Au-Pd alloy. The X-ray diffraction (XRD) measurements were performed with a X-ray diffractometer (Bruker DRX-Linxeye) using Cu Kα radiation (λ = 1.540 Å), in a 2θ range from 25º to 70º. Distances between peaks were compared to the JCPDS 5-0664 of the International Center for Diffraction Data to determine crystalline structures. Chemical characterization was performed by Fourier transformed infrared spectroscopy (FT-IR) with a Perkin-Elmer 2000 FT-IR spectrometer. Measurements were performed with pressed discs made using potassium bromide (KBr) powder. The FT-IR spectra were collected between the wavenumber of 4000-400 cm^{-1} with one hundred scans accumulated per sample. Surface chemical composition analysis was performed by X-ray photoelectron spectroscopy (XPS) using an ESCALAB 200A, VG Scientific (UK) with PISCES software for data acquisition and analysis. For that purpose, an achromatic Al (Kα) X-ray source operating at 15 kV (300 W) was used. Spectra analysis was performed using peak fitting with Gaussian-Lorentzian peak shape and Shirley type background subtraction (or linear taking in account the data). As a final analysis, zeta potentials were determined from streaming potential measurements with a commercial electro kinetic analyzer (EKA) (Anton Paar GmbH, Austria). The streaming potential was measured using Ag/AgCl electrodes installed at both ends of the streaming channel. The electrolyte used was 1 mM KCl and the experiments were performed at 25ºC. The mashed composites were placed on a cylindrical powder cell and the streaming potential was measured while applying an electrolyte flow in alternating directions and pressure ramps from 0 to 200 mbar. For each material, an average of at least three measurements was reported (three in each flow direction).

2.3. Microorganisms and culture conditions

The microorganisms used in this study were *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. Microorganisms were kept at -70ºC in a

solution containing 70% tryptic soy broth (TSB; Liofilchem, Italy) and 30% diluted glycerol. For each experiment the bacteria were freshly prepared by inoculating the frozen bacteria suspension onto nutrient agar (NA; Liofilchem, Italy) for 24 h at 37ºC. Stationary phase cells were obtained by incubating two to three colonies, from the NA, in 5 mL of TSB for 18h at 37ºC and 150 rpm.

2.4. Antibacterial activity of nanoHA-ZnO composites

To study the antimicrobial activity of nanoHA-ZnO composites, changes in the growth of each bacterium, incubated in TSB, in the presence of the composites were investigated. Overnight cultures, containing the bacteria on stationary phase, were inoculated into 2 mL of fresh TSB, in order to obtain an initial suspension of 10^5 colony forming units (CFU) mL^{-1}. The composite samples and pure nanoHA (control material), previously sterilized by dry-heat (180ºC, 1 h), were added to the test tubes and incubated in a constant-temperature shaker at 150 rpm and 37ºC. A tube without any biomaterial was used as a positive control. Negative controls were obtained by incubating the biomaterial in TSB without adding any bacterial cells. After 24 h of bacteria culture incubation, the optical density of these suspensions was measured on a microplate reader (Stat Fax 3200, Awareness Technology Inc.) at 545 nm and the background (turbidity due to growth medium and light-scattering properties of the nanoparticles) was eliminated by taking blank readings. Three replicate tubes were prepared for each material sample.

2.5. Biofilm formation on nanoHA-ZnO composites

The anti-adhesive potential of nanoHA-ZnO composites was evaluated by biofilm formation studies. Overnight TSB cultures containing the relevant bacterial strain were used to prepare a suspension with a turbidity equivalent to 0.5 McFarland standard (ca., 10^8 CFU mL^{-1}), then diluted in TSB to 1/100 (obtaining a 10^6 CFU mL^{-1}). The composite samples and pure nanoHA (control material), formerly sterilized by dry-heat (180ºC, 1 h), were placed in tubes with 2 mL of the bacterial suspension previously prepared and incubated at 37°C and 150 rpm. At the end of 24 h incubation, each material samples was rinsed twice with phosphate-buffered saline (PBS) and transferred

to tubes with 5 mL of sterile PBS and sonicated for two seconds, at 20 KHz in a sonicator (Sonopuls HD 2200, Bandelin, Germany) with a MS 73 probe. Serial dilutions of the sonicated solutions were prepared and inoculated in NA. The number of colonies was counted after overnight incubation at 37ºC and bacterial adhesion on the samples surface was expressed as number of adherent bacteria per mm^2. Experiments were done in triplicate.

2.6. Statistical analysis

Experimental data was analyzed using IBM® SPSS® Statistics (vs. 19.0, SPSS, USA). The mean values were calculated and reported as the mean ± standard deviation (n=3). The one-way analysis of variance (ANOVA) followed by the *post hoc* Tukey HSD multiple comparison test was used to determine the significant difference ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Physicochemical characterization of nanoHA-ZnO composites

SEM images provide the direct observation of the composites surface morphology and, particularly, the back scattering images highlight the appearance of ZnO particles at the nanoHA surface (Fig.1). The primary ZnO particles tend to aggregate to form larger secondary particles uniformly disperse on nanoHA matrix. The ZnO nanoparticles are prone to aggregate and this may happen due to the large specific surface area [8, 26].

The 2 wt % ZnO (<100 nm) composite heat-treated at 1000ºC was chosen to illustrate the crystal structure of the ZnO particles in the nanoHA matrix (Fig. 2). The results of XRD analysis for other composite samples were similar to the one above referred, thus they are not presented. The characteristic diffraction peaks (100), (002), (101), (102), (110) and (103) are observed in all nanoHA-ZnO composites and they can be indexed to hexagonal ZnO (International Center for Diffraction Data, JCPDS 5-0664). The presence of nanoHA phase is clearly recorded in all composite samples. The strong intensity and narrow width of diffraction peaks indicate that the resulting products were highly crystalline.

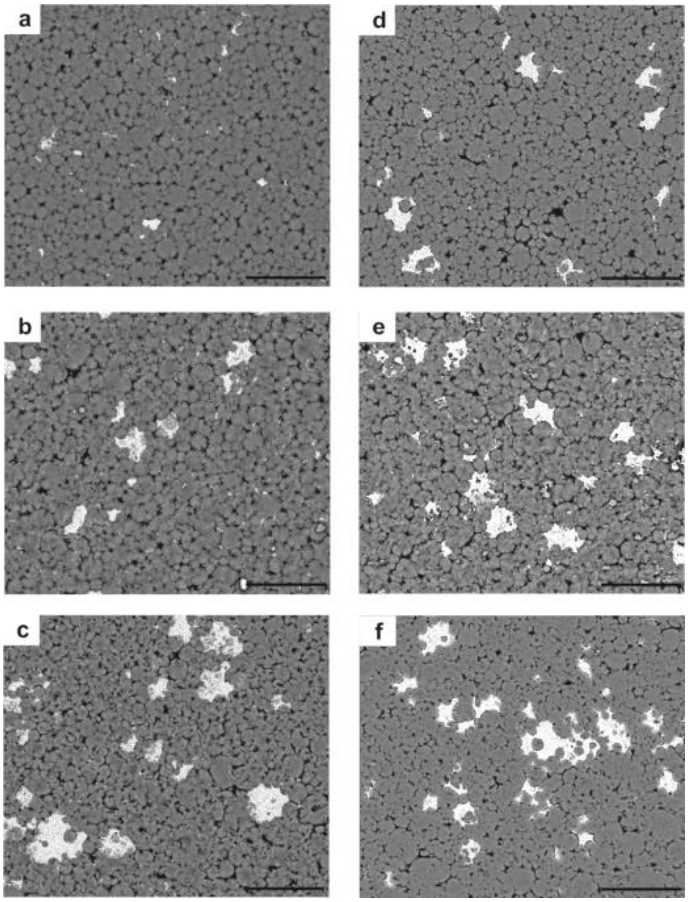

Figure 1. Back scattering electrons images collected by SEM of nanoHA-ZnO composites with different amounts of ZnO (<100 nm): 2 wt % (a, d), 10 wt % (b, e) and 25 wt % (c, f), and heat-treated at two different temperatures: 830ºC (a-c) and 1000ºC (d-f). Scale bar 20 µm.

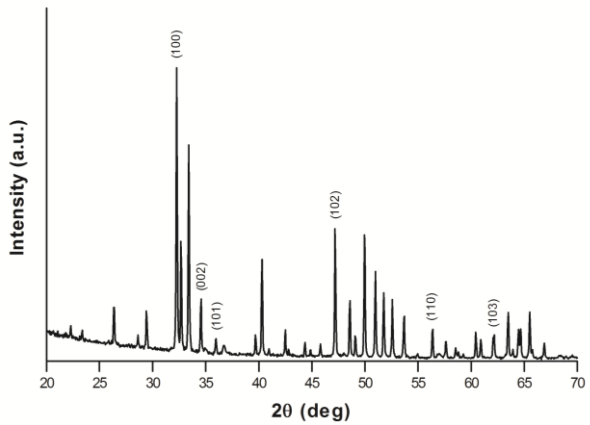

Figure 2. XRD pattern of nanoHA-ZnO composite with 2 wt % ZnO (<100 nm), heat-treated at 1000ºC.

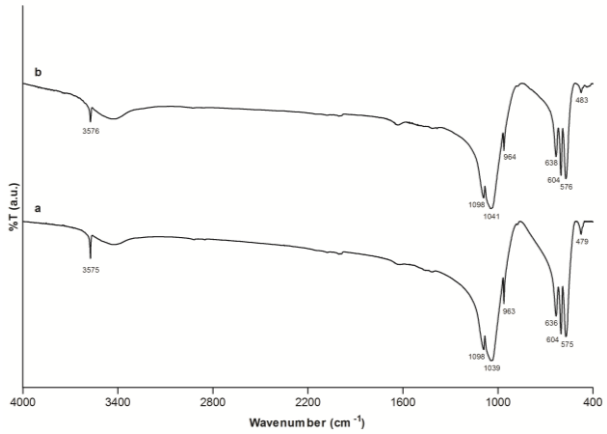

Figure 3. FT-IR spectra of pure nanoHA (a) and nanoHA-ZnO composite with 10 wt % ZnO (<100 nm) (b), both heat-treated at 830ºC.

Figure 3 depicts the FT-IR spectra of pure nanoHA and nanoHA-ZnO composite with 10 wt % ZnO (<100 nm), both heat-treated at 830ºC. Comparable results were obtained for the other composites samples and heat-treatment (data not shown). It may be seen that the major peaks of nanoHA associated with PO_4^{3-} (1098, 1039, 963, 604, 575 and 479 cm^{-1}) and OH^- (3575 and 636 cm^{-1}) appear on both plots (Fig. 3 (a,b)). Compared with pure nanoHA, a new small absorption band in the range of 479 - 400 cm^{-1} was found in the FT-IR spectra of nanoHA-ZnO composite (Fig. 3(b)), which was correlated to the stretching vibration of ZnO [27]. FT-IR and XRD results confirmed that heat-treated nanoHA-ZnO composites were phase pure and crystalline.

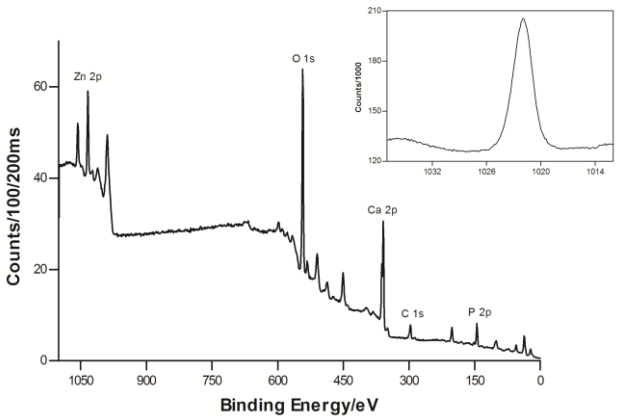

Figure 4. XPS wide scan spectra of nanoHA-ZnO composite with 10 wt % ZnO (<100 nm) heat-treated at 830ºC and the detected Zn 2p peak (inset) in the composites surfaces.

The antimicrobial activity strongly depends on the structure and stability of the different surface planes of ZnO, as well as the number of defects on the ZnO particles surfaces. Thus, XPS was used to study the ZnO particles in the nanoHA matrix once is a technique highly sensitive to the chemical composition and the environments of the elements in the material [10, 28]. The XPS wide scan spectra of nanoHA-ZnO composite (Fig. 4) shows the presence of Zn 2p, O 1s, Ca 2p, C 1s and P 2p core levels. The high resolution spectra of Zn 2p level is shown in Figure 4 (inset). The Zn peaks in the different surfaces are all in the same position and the binding energy peak of Zn at 1022 eV belongs to ZnO.

Table I. Zeta potential for nanoHA-ZnO composites with ZnO particle size <100 nm, heat-treated at 830 and 1000ºC

Sample (ZnO wt %)	**Zeta Potential (mV)**	
	830ºC	**1000ºC**
0%	-15.8 ± 2.0	-19.2 ± 1.1
2%	-16.9 ± 2.0	-15.9 ± 1.2[a]
10%	-16.4 ± 1.3	-11.2 ± 1.6[a]
25%	-12.0 ± 0.8[a]	-9.6 ± 0.4[a]

[a] Significantly different from pure nanoHA (0%) ($p < 0.05$)

Table I encloses the average zeta potential of pure nanoHA (0%) and nanoHA-ZnO composites (with ZnO particle size <100 nm). All materials showed to be negatively charged after both heat-treatments. The presence of ZnO particles might be the main factor responsible for the observed change of zeta potential. The higher the ZnO amount, the less negatively charge will be the composite.

3.2. Antibacterial activity of nanoHA-ZnO composites

The effect of various nanoHA-ZnO composites on the growth of *S. aureus* and *E. coli* is presented in Figure 5.

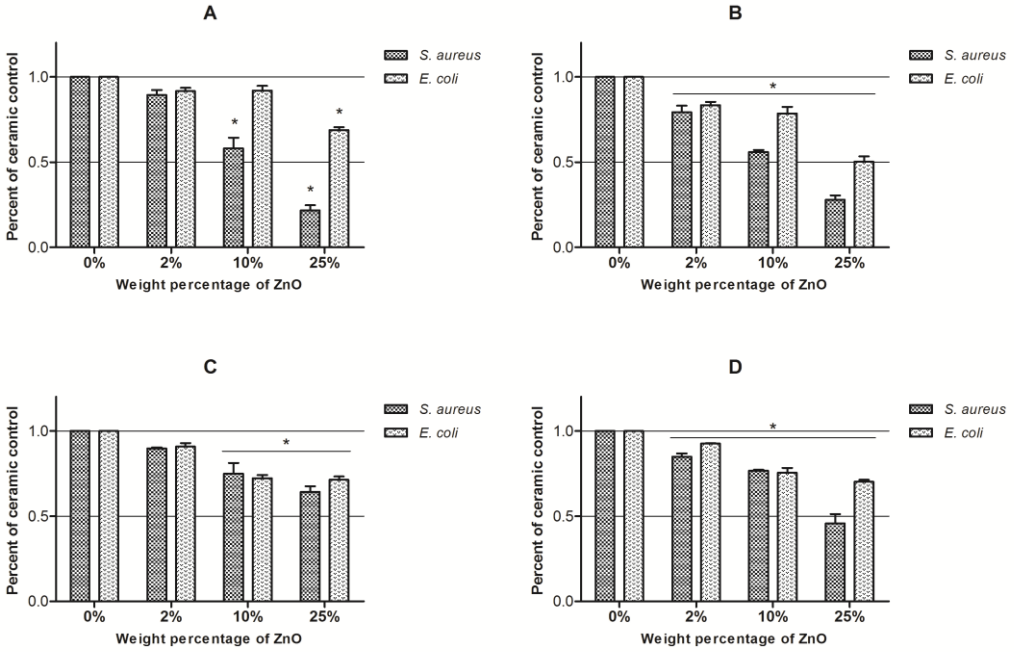

Figure 5. Bacteria population (as a percentage of population on ceramic control) determined by optical density readings of *S. aureus* and *E. coli* suspension cultured for 24 h with the composite samples at various weight percentages and with different ZnO particle size:<100 nm (A and C) and <50 nm (B and D). Material samples were heat-treated at 830ºC (A and B) and 1000ºC (C and D). *p < 0.05, significant reduction compared to pure nanoHA (0%), according to Tukey HSD.

Optical density data after 24 h of incubation indicated a significant reduction in total bacteria counts for bacteria cultured in the presence of the composites compared to bacteria cultured in the presence of pure ceramic samples. Similar results were obtained for composites with ZnO particle size of <100 nm and <1 μm (data not shown). These results also indicated that antibacterial efficiency increases with increasing ZnO amount for both studied bacteria. The data clearly suggest that, in comparison with pure nanoHA, composites formulated with ZnO nanoparticles with smaller sizes (<50 nm) showed a significant growth inhibition at just 2 wt %, whereas for composites with relatively larger ZnO particles (<1 μm and <100 nm) the same effect was just observed at 10 - 25 wt % (depending on the particular bacterial strain). Similar profiles of antibacterial activity were obtained for both heat-treatments conditions.

3.3. Biofilm formation on nanoHA-ZnO composites

The anti-adhesive properties of nanoHA-ZnO composites were also evaluated, since biofilm formation on materials surface is an undesired situation [6, 7]. With that propose, the bacteria were grown in contact with composite samples, with three different ZnO particle sizes and two distinct heat-treatments. After 24 h de number of viable cells attached on composite surface was evaluated. The obtained results for *S. aureus* and *E. coli* are shown in Figure 6 and 7, respectively.

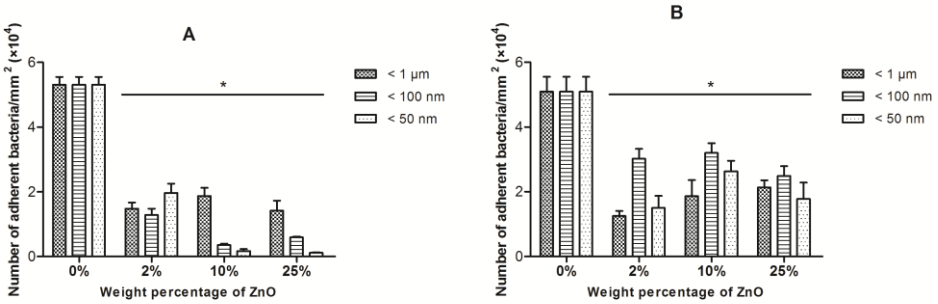

Figure 6. Biofilm formation of *S. aureus* on composite samples, at various weight percentages and different ZnO particles size, as determined by CFU mm^{-2}, after 24 h of incubation. Material samples were heat-treated at 830ºC (A) and 1000ºC (B). All composite samples showed a significant reduction compared to pure nanoHA (0%) (*p<0.05, according to Tukey HSD).

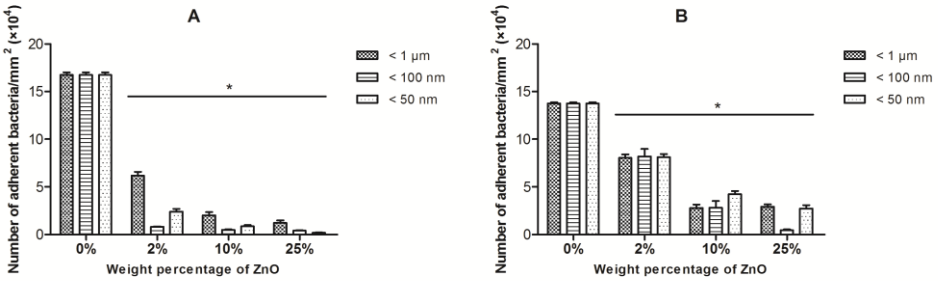

Figure 7. Biofilm formation of *E. coli* on composite samples, at various weight percentages and different ZnO particles size, as determined by CFU mm^{-2}, after 24 h of incubation. Material samples were heat-treated at 830ºC (A) and 1000ºC (B). All

composite samples showed a significant reduction compared to pure nanoHA (0%) (*p<0.05, according to Tukey HSD).

Compared with pure nanoHA, a strong and significant reduction on the biofilm formation was noticed, for the various composites. The reduction in biofilm formation was probably related to the reduction in the number of viable bacteria presented at the material surface. For all composites heat-treated at 830ºC, the reduction observed was above 60%, for both tested bacteria (86% reduction in biofilm formation, on average). The inclusion of 25 wt % ZnO (<50 nm) reduced biofilm growth by ~98% for *S. aureus* and 99% for *E. coli*, when compared to pure nanoHA (materials heat-treated at 830ºC). The composites heat-treated at 1000ºC also display a significant biofilm inhibition (62% reduction in biofilm formation, on average) nevertheless, the materials heat-treated at 830ºC display higher anti-adhesive effect. Sintering at 1000ºC may have established stronger bonds, leading to ZnO particles entrapment by nanoHA matrix. Moreover, these results also suggested that the composite inhibitory effect had higher effect against *S. aureus* adhesion than against *E. coli*, in consistent with previous reports [19, 22] still, other studies observed the opposite effect [9, 29]. This differential effect may be attributed to different structure, chemical composition and thickness of the cell wall between *S. aureus* and *E. coli* [18, 22] as well as ZnO mechanism of action.

The results obtained are in agreement with previous works reporting that ZnO particles are effective in inhibiting both Gram-positive and Gram-negative bacteria and the antibacterial properties of ZnO particles depends on the concentration and surface area [9, 11, 13, 15-18, 22, 29, 30]. This reduced bacteria activity may be due to a variety of mechanisms, including, the production of highly reactive oxygen species (ROS) (O_2^{2-}, OH^- and H_2O_2) on the surface of ZnO particles connected with fatal damage to the bacteria [9, 22]. However, several studies reported that the generation of H_2O_2 from the surface of ZnO is one of the primary effects that contribute to the antibacterial effect, which takes place via penetration of H_2O_2 through the cell walls. Because the hydroxyl radicals and superoxides are negatively charged particles, they cannot penetrate into the cell membrane and must remain in direct contact with the outer surface of the bacteria; on the other hand, H_2O_2 can penetrate into the cell [10, 16]. In addition, the generation of H_2O_2 strongly depends on the specific surface area of ZnO,

which results in higher antibacterial activity of the smaller nanoparticles [9, 10, 22]. These evidences can explain the strong effect observed in this study for composites with ZnO nanoparticles (<50 nm). Furthermore, the increase of ZnO concentration in the composite results in a larger amount of H_2O_2 generated. The antibacterial activity exhibited by ZnO nanoparticles could also be due to the presence of soluble Zn^{2+} formed when ZnO is suspended in solution. The solubility of metal oxide nanoparticles increases as their particle size decreases, and the enhanced activity of the smaller sized ZnO nanoparticles could also be due to the formation of dissolved Zn^{2+} ions [8]. Another possible explanation for the antibacterial effect is based on the abrasive surface texture of ZnO due to surface defects, which may cause damage and disorganization in the cell wall [10, 15]. And, if for one hand the anti-adhesive resistance of the bacteria to nanoHA-ZnO composites (Figs. 6 and 7) might be caused by an electrostatic repulsion resulting from identical charges at the surface of the composites (Table I) and bacteria (the overall charge of the bacteria at biological pH values is negative, because of the excess number of carboxylic and other groups [31]) which induced repulsion and prevented the contact and further adhesion [32]; it is also possible, on the other hand, that the ceramic composites surfaces might slightly degrade during the incubation period and some ZnO particles were released into the culture medium interfering with bacterial cell membranes, due to electrostatic attraction between ZnO particles and bacteria surface [20]. The above hypothesis may explain the great anti-adhesive properties display by the composites samples and also may explain the differences observed between both heat-treatments.

4. CONCLUSION

Implant infection remains as one of the major and often irreducible problems associated to the clinical use of biomaterials, demanding new therapeutic and preventive strategies. In this study the antimicrobial activity of ZnO powders was assessed, with particle sizes ranging from microns to nanometers, and at different weight percentages, when integrated into nanoHA. It was found that ZnO nanoparticles (<50 nm) showed to be more effective, at a lower weight percentage, than the composites with other particles sizes, for both bacterial species and heat-treatments tested. The enhanced bioactivity of

smaller particles may be attributed to the higher surface area to volume ratio, which results in the generation of a larger number of active oxygen species which may cause fatal damage to microorganisms. Therefore, composites of nanoHA with ZnO nanoparticles should be further studied as a potential material to prevent microorganisms from attaching, colonizing, spreading, and forming biofilms in medical materials.

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CHAPTER III

Antibacterial activity and biocompatibility of three-dimensional nanostructured porous granules of hydroxyapatite and zinc oxide nanoparticles – An *in vitro* and *in vivo* study

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Submitted

ABSTRACT

Ceramic scaffolds are widely studied in the bone tissue engineering field due to their potential in regenerative medicine. However, adhesion of microorganisms on biomaterials with subsequent formation of antibiotic-resistant biofilms is a critical factor in implant-related infections. Therefore, new strategies are needed to address this problem. In the present study, three-dimensional and interconnected porous granules of nanostructured hydroxyapatite (nanoHA) incorporated with different amounts of zinc oxide (ZnO) nanoparticles were produced using a simple polymer sponge replication method. As *in vitro* experiments, granules were exposed to *Staphylococcus aureus* and *Staphylococcus epidermidis* and, after 24 h, the planktonic and sessile populations were assessed. Cytocompatibility towards osteoblast-like cells (MG-63 cell line) was also evaluated for a period of 1 and 3 days, through resazurin assay and imaging flow cytometry analysis. As *in vivo* experiments, nanoHA porous granules with and without ZnO nanoparticles were implanted into the subcutaneous tissue in rats and inflammatory response after 3, 7 and 30 days was examined, as well as their antibacterial activity after 1 and 3 days of *S. aureus* inoculation. The developed composites showed to be especially effective at reducing bacterial activity *in vitro* and *in vivo* for a weight percentage of 2% ZnO, with a low cell growth inhibition *in vitro* and no differences in the connective tissue growth and inflammatory response *in vivo*. Altogether, these results suggest that nanoHA-ZnO porous granules have a great potential to be used in orthopaedic and dental applications as template for bone regeneration and, simultaneously, to restrain biomaterial-associated infections.

Keywords: nanostructured hydroxyapatite, zinc oxide nanoparticles, porous granules, antibacterial activity, biocompatibility

1. INTRODUCTION

In bone tissue engineering, the search for bone substitutes is still a challenge to researchers. In this framework, ceramic scaffolds are widely studied and are a key component providing support to cell adhesion, proliferation and, ultimately, ensuring structural support to the newly formed tissue [1, 2]. Macroporous structured granules are particularly useful, since those materials can fill irregularly shaped bone defects and patient-specific shapes, in orthopaedic or periodontal applications [3]. Among the various forms of calcium phosphate ceramics, hydroxyapatite (HA) has received considerable attention due to its excellent bioactive and osteoconductive properties as it bonds to bone and enhances bone tissue formation. Nanocrystalline synthetic HA in particular has superior functional properties due to its particle size, large surface area to volume ratio, reactivity, and biomimetic morphologies compared to the mineral phase of bone [4, 5]. However, rapid host protein adhesion to the implant and low vascularity in the area of trauma create a suitable environment for bacterial adherence and, as a result, implant surfaces can harbour bacterial infections in the form of biofilm [6]. Due to the increasing number of prostheses being implanted every year, infection of implanted material is a major complication for modern medicine, which results in high socioeconomic costs since the treatment is often prolonged and expensive. Extensive antibiotics treatment alone is usually only able to suppress, but not eliminate these infections because biofilm state protects the bacteria against antibiotics and host immune system, thus making revision surgery mandatory. However, each revision surgery bears an increased risk of yet another infection [7, 8]. In clinical practice, current strategies for the prevention and treatment of these infections include the use of antibiotic-loaded cements or localized antimicrobial delivery systems. However, one of the main drawbacks of these systems is the elution kinetics which usually exhibits a burst release of the adsorbed antibiotics in the first hours followed by a long-lasting phase of slow release at very low concentrations, below the minimal inhibitory concentration. Such behaviour indulges the emergence of drug-resistant bacterial strains. Moreover, antimicrobials are typically ineffective in penetrating pre-established biofilms [9]. As a result, there is an urgent clinical need to develop alternative infection-resistant surfaces, beyond the use of antibiotics, as the main strategy to prevent the establishment of implant colonization and biofilm formation. In this regard, inorganic

materials like metal oxide are gaining significant scientific attention compared to conventional antibacterial agents [10-12]. Among them, zinc oxide (ZnO) is a prominent agent exhibiting a broad spectrum of antimicrobial activity [13]. Moreover, smaller particles, at nanoscale dimensions, with higher surface area, have further enhanced the antibacterial behaviour of ZnO [12, 14, 15]. Particularly, the authors' previous report confirmed that two-dimensional nanoHA samples incorporated with ZnO (particles from the micrometer scale down to the nanometer range) had robust *in vitro* antibacterial activity, with an inverse relationship between particle size and antibacterial effect [16]. As a consequence, the challenge of the present study was the continued investigation of the natural antibacterial properties of ZnO nanoparticles when incorporated, at different concentrations, into nanostructured hydroxyapatite (nanoHA) for the development of scaffolds, specifically three-dimensional nanostructured porous granules. *In vitro* antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* was investigated, as both strains are known to be leading species of implant-related infections [8, 17]. In addition, the cytocompatibility of such composites were also evaluated towards osteoblast-like cells (MG-63). Further, using a subcutaneous rat insertion model, the inflammatory reaction and the *in vivo* antibacterial activity of nanoHA-ZnO granules were also investigated.

2. MATERIALS AND METHODS

2.1. Preparation of nanoHA-ZnO granules

In order to obtain the nanoHA-ZnO porous granules, the polymer sponge replication method was adopted [18]. Initially, the composite powder were prepared by mixing as-received ZnO nanoparticles (<50 nm particle size, Sigma-Aldrich) with nanoHA powder (nanoXIM·HAp202; Fluidinova S.A., Portugal) at weight percentages (wt %) of 0, 0.5, 1, and 2. Then, four different slurries were prepared using the ratio of 5:4.5:0.2 for powder (g), water (mL), and deflocculating agent (mL), respectively. Deflocculating agent (Dolapix CE-64, Zschimmer & Schwarz, Germany) was used for the composite suspension to reach the appropriate viscosity [19]. Polyurethane sponges, used as template, were impregnated with the slurries, gently squeezed to remove the exceeding suspension and dried at room temperature for 24 h. The infiltrated sponges

were heat-treated according to the following sintering cycle: heating rate of 1ºC/min with 1 hour dwelling time at 600ºC for the polyurethane sponge to burn out, followed by a heating rate of 4ºC/min with 1 hour dwelling time at 830ºC [16, 20]. The samples were cooled inside the furnace. Afterward, the scaffolds were cut with a sharp razor and sieved to obtained granules with sizes between 2 to 3 mm. Prior to biological experiments, the materials were sterilized by dry heat (180ºC, 2 h).

2.2. Granules morphology

The morphology of nanoHA porous granules with and without ZnO nanoparticles was characterized by scanning electron microscopy (SEM, FEI Quanta 400 FEG ESEM) operated at 15 kV, and coupled to an energy dispersive X-ray analyzer (EDAX Genesis X4M) for elemental analysis. Thus, the granules were attached with Araldite^{TM} to an aluminium sample holder and sputter-coated with a gold-palladium conductive film (SPI-Module).

2.3. Measurement of Zn-ion release from granules surfaces

In order to measure the amount of zinc (Zn^{2+}) released from the biomaterials surfaces, granules of pure nanoHA and nanoHA with 2% ZnO were immersed in ultra pure water for 1 and 3 days. After incubation, the supernatants were collected and analyzed by atomic absorption spectroscopy (AAS, Hitachi-Z8200 Polarised Zeeman). The ion concentrations were reported in units of parts per million (ppm).

2.4. *In vitro* antibacterial activity

In vitro antibacterial activity of nanoHA granules with and without ZnO was quantitatively evaluated against *S. aureus* ATCC 49230 and *S. epidermidis* ATCC 35984 on 96-well plates. Initially, overnight cultures were diluted in tryptic soy broth (TSB, Liofilchem) and an aliquot of 200 µL of this bacterial suspension (10^4 colony forming units (CFU)/mL) was added to each well, containing 10 mg of granules. After 24 h incubation at 37ºC, the bacterial suspension, from each well, was collected and the planktonic bacteria were quantified by optical density (OD) at 545 nm with a microplate reader and by colony counts (CFU/mL), on nutrient agar (NA, Liofilchem)

plates. Then, the materials were carefully washed twice with saline solution (0.9% NaCl, JT Baker), to remove non-adherent bacteria, and the metabolic activity of sessile bacteria was assessed by Alamar Blue (AB) method. Briefly, 200 µL of TSB with 10% resazurin (Sigma-Aldrich) were added to each well and incubated for 3 h at 37ºC. AB is a redox indicator that both fluoresces and changes colour in response to chemical reduction. The extent of reduction is a reflection of bacterial metabolic activity. After incubation, the fluorescence was recorded using a microplate reader (excitation: 530 nm and emission: 590 nm). Five samples per group were used.

2.5. *In vitro* cytocompatibility test

For the *in vitro* cytocompatibility study, human osteoblast-like cells lineage (MG-63) were maintained in alpha minimum essential medium (α-MEM, Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco), 1% (v/v) penicillin-streptomycin (100 IU/mL penicillin and 2.5 µg/mL streptomycin, Gibco), and 1% amphotericin B (2.5 µg/mL, Gibco). Incubation was carried out in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC. Prior cell seeding, 10 mg of granules of pure nanoHA and nanoHA with 2% ZnO were pre-incubated into 24-well plate, with complete medium for 1 h at 37ºC. Then, the medium was removed and the cells were seeded on the granules at a density of $5x10^4$cell/well. Subsequently, the culture plate was incubated for 1 and 3 days. To evaluate the cellular metabolic activity, cells were incubated with resazurin solution (0.1 mg/mL, Sigma-Aldrich) for 3 h at 37ºC. Afterwards, the supernatant had the fluorescence intensity measured in a fluorimeter (excitation: 530 nm and emission: 590 nm, Synergy HT, BioTek). The experiment was performed in triplicate for each group.

2.6. Apoptosis assay by Imaging Flow Cytometry

The cytotoxic nature of nanoHA-ZnO granules was also studied. Thus, MG-63 cells were seeded at concentration of 10^6 cells/mL into 6-well culture plate. After 24 h (attachment phase), granules of pure nanoHA and nanoHA with 2% ZnO (10 mg) were added over the cells. Tissue-culture polystyrene (TCPS) wells, without any biomaterial, were used as negative control. Three replicas for each group were used. The following

incubation was carried out for 1 and 3 days. Afterwards, culture medium (containing dead cells) and adherent cells (detached with 0.5% of trypsin) were collected and the apoptosis assay was done according to the manufacturer (Annexin V-FITC Apoptosis Detection Kit, Sigma-Aldrich). Briefly, 10^6 cell/mL were suspended in buffer with propidium iodide (PI, 0.5 mg/mL) and Annexin V-FITC solution (100 mg/ mL) at 37ºC for 30 min, in absence of light. The cells were immediately analyzed with imaging flow cytometry (Image Stream X Mark II, Amnis), by detecting the fluorescence emitted by PI (DNA - dead cells) and Annexin V (phosphatidylserine - apoptotic cells) in each event. A single-cell gate was used to exclude aggregated cells (doublets, triplets) and 20 000 gated events were collected for each analysis.

2.7. *In vivo* biocompatibility and infected pouch studies

Forty male Wistar rats between 150 and 250 g (8 week-old, Charles River, Wilmington, MA) were employed for subcutaneous implantation of biomaterials (20 animals were used for the inflammatory response and further 20 animals for the *in vivo* infected pouch with *S. aureus*). The studies were performed in accordance with Animal Ethical Committee and fulfilled all legal requirements (Process number 016825, DGV, Lisbon, Portugal). Surgical procedures were performed under standard aseptic conditions. Pre-operatively, rats were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Then, the dorsal area of each rat was shaved, disinfected and a 2-4 cm midline incision was created. Sharp and blunt dissections were used to develop two individual subcutaneous pockets distributed on the left and right side of the incision. The sterilized granules of pure nanoHA and nanoHA with 2% ZnO were moistened with physiologic saline solution (0.9% NaCl) and inserted bilaterally into the subcutaneous pockets. The dorsal incision was then closed with surgical staples. Post-operatively, the rats were subcutaneously administered saline solution (10-20 mL/kg) for fluid replacement. After recovery, the rats were housed in pairs and allowed to move in their cages without restriction. They were fed with commercial rat chow and water *ad libitum*. Three days, one and four weeks after surgery, the rats were euthanized with carbon dioxide asphyxiation. All samples (biomaterial and surrounding tissue) were explanted and gross examination was performed on each biopsy prior to fixation for histological analysis.

Same surgical procedures were conducted for the infected pocket model. Thus, after biomaterial implantation and closing the injury, pockets were inoculated with 0.5 mL of a bacterial suspension of *S. aureus* ATCC 49230 at 10^4 CFU/mL, in saline solution. The recovery and post-surgery treatments were done as described above. After 1 and 3 days, the animals were euthanized as aforementioned and the biomaterials were carefully harvested with surrounding tissue under sterile conditions. The specimens were homogenized in saline solution for bacterial quantification. For that purpose, serial dilutions were made and inoculated onto NA plates. After 24 h of incubation at 37ºC, bacterial colonies were counted and the CFUs/mL were calculated.

2.7.1. Histological analysis

The collected specimens were fixed in 10% neutralized buffered formalin for 24 h and then processed using a standard method. Briefly, fixed specimens were embedded in paraffin and were sectioned longitudinally with a microtome (5-µm of thickness). The sections were stained with hematoxylin-eosin (HE) for light microscopy examination.

2.8. Statistical analysis

Results were expressed as mean ± standard deviation. Statistical analysis was assessed by one-way analysis of variance (ANOVA), followed by multiple comparisons among groups using the Tukey HSD *post hoc* test (IBM® SPSS® Statistics, vs. 19.0). The data were considered to be significantly different when $p < 0.05$ was obtained.

3. RESULTS

3.1. Granules morphology

The nanoHA granules, with and without ZnO nanoparticles, presented a heteroporous morphology with macro and micro-pores as well as vast pore interconnectivity under SEM visualization (Figs. 1A and B). High magnification images showed that nanoHA crystals aggregates, after the sintering treatment, strongly bond to each other and yet preserving the nano-size dimension (Fig. 1 C). The presence of ZnO

nanoparticles, as lighter spots, homogeneously dispersed in nanoHA substrate was highlight through backscattered electrons imaging (Fig. 1D) and further confirmed by EDAX spectra showing the presence of elemental Zn peak (Fig. 1E).

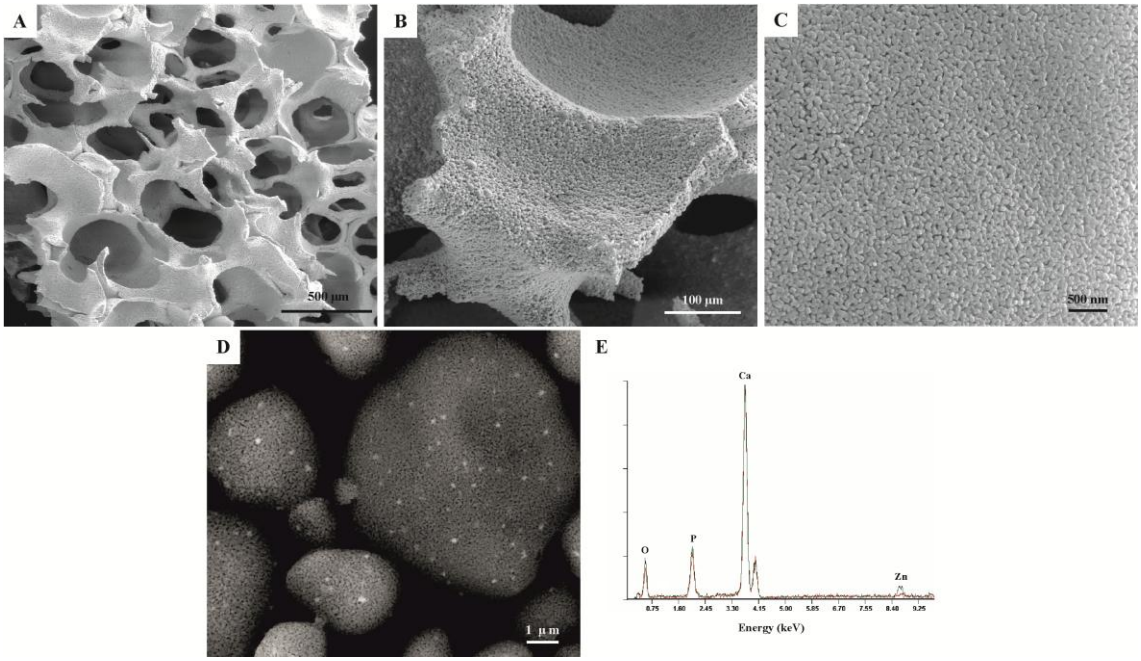

Figure 1. Micro- and nano-structure of porous granules of nanoHA (A, B and C) and nanoHA with 2% ZnO (D) obtained by the polymer replication method. SEM images obtained by secondary electrons (A, B and C) and by backscattered electrons, at higher magnifications, to highlight ZnO nanoparticles dispersion on nanoHA substrate (lighter spots) (D), additionally confirmed by EDAX spectrum (E). Scale bar 500 µm (A), 100 µm (B), 500 nm (C) and 1 µm(D).

3.2. Zn-ion released from granules surfaces

Elemental zinc released from granules surface of pure nanoHA and nanoHA with 2% ZnO, when immersed in ultrapure water, was measured by AAS. Results indicated that, for composite samples, the zinc amount slightly increased from 0.30 to 0.38 ppm, from day 1 to day 3, respectively. For granules of pure nanoHA, as expected, zinc-ion was not detected in this supernatant.

3.3. *In vitro* antibacterial activity

Prior to *in vivo* experiments, the *in vitro* antibacterial efficacy of nanoHA-ZnO porous granules was first validated. For this, the materials were inoculated with *S. aureus* and *S. epidermidis* and their bactericidal efficacies against planktonic and sessile bacterial growth were assessed, after 24 h incubation. Regarding *S. aureus* planktonic values, either by OD or CFU/mL, only nanoHA impregnated with 2% ZnO exhibited statistically significant antibacterial activity, with no bacterial growth on the culture plates (Fig. 2). Whereas for *S. epidermidis* a significant reduction in planktonic growth, assessed by agar plating, was observed for the three tested composites, when compared to pure nanoHA granules (Fig. 2). Furthermore, the metabolically active bacteria on material surface (sessile population) exhibited a concentration-dependent effect for both microorganisms (Fig. 3), with a significant reduction for the composites with 1 or 2% ZnO nanoparticles, comparatively to pure nanoHA granules. Noteworthy, the granules with 2% ZnO have enabled a complete inhibition of bacterial viability and therefore this was the composite selected for the subsequent experiments.

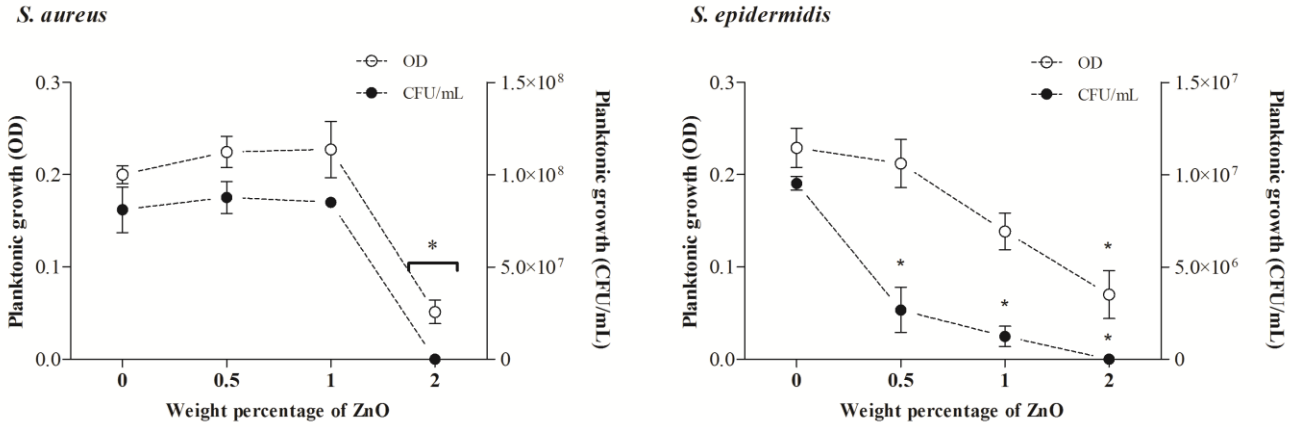

Figure 2. Planktonic population of *S. aureus* and *S. epidermidis* cultured for 24 h with porous granules of nanoHA (control) and nanoHA with different amounts of ZnO nanoparticles. Quantification by OD and CFU/mL. *$p < 0.05$, significant reduction compared to control (0%).

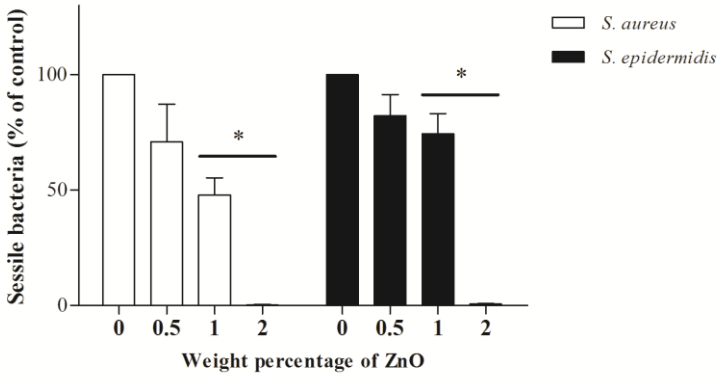

Figure 3. *S. aureus* and *S. epidermidis* sessile population on nanoHA granules with different amounts of ZnO nanoparticles (as a percentage of sessile population on control), after 24 h incubation. Quantification by AB method. *$p < 0.05$, significant reduction compared to control (0%).

3.4. *In vitro* cytocompatibility test

The metabolic activity of MG-63 cells on porous granules of pure nanoHA and nanoHA with 2% ZnO nanoparticles was assessed by resazurin assay and is shown in Figure 4. The resazurin values of cells cultured with the composite were similar to those obtained for pure nanoHA granules after 1 day of culture. However, at day 3, the nanoHA-ZnO granules caused an inhibitory effect on MG63 cells, with a lower increase on cell metabolic activity, compared to the control.

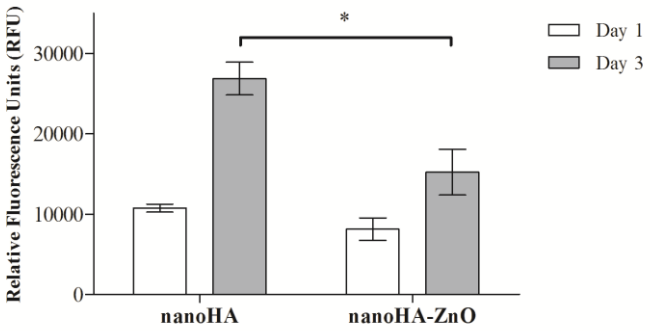

Figure 4. Metabolic activity of MG-63 cells cultured for 1 and 3 days on porous granules of pure nanoHA and nanoHA with 2% ZnO nanoparticles, estimated by resazurin assay. *$p < 0.05$, significantly different.

3.5. Apoptosis assay by Imaging Flow Cytometry

To further confirm the mode of cell death induced by nanoHA-ZnO granules, MG-63 cells exposed to the materials for 1 and 3 days were analyzed by imaging flow cytometry using PI/Annexin V-FITC staining. As depicted in table I, nanoHA granules had no negative effect on osteoblasts. In fact, cells were more viable on this material when compared to control (TCPS) with low or even negligible percentage of apoptotic or dead cells. On the other hand, the cells incubated with nanoHA-ZnO granules had started the apoptotic process, expressed by the higher percentage of cells in early and late apoptosis, comparatively to the other groups. However, it is important to underline that, after 3 days of culture, the percentage of apoptotic cells, as well as dead cells had shown a tendency to decline along with an increase in the number of living cells.

Table I: Apoptosis assay evaluated by image flow cytometry

Day	**Control (TCPS)**				**nanoHA**				**nanoHA-ZnO**			
	Live	EA	LA	Dead	Live	EA	LA	Dead	Live	EA	LA	Dead
1	88.4	0.7	1.5	9.3	99.5	0.2	0.0	0.3	79.6	13.6	3.1	3.4
3	99.3	0.7	0.0	0.0	99.5	0.5	0.0	0.0	86.8	12.2	0.0	0.8

EA: Early apoptosis; LA: Late apoptosis

3.6. *In vivo* biocompatibility and infected pouch studies

It should be noted that all surgical procedures performed went through without any accidents, and the animals recovered uneventfully from surgery and general anaesthesia. Their post-operative recovery was normal, and all surgical sites healed without complications.

The *in vivo* biocompatibility evaluation of nanoHA granules with and without ZnO revealed further features than the *in vitro* analyses. During the explant of the subcutaneous implants intense new vascularisation could be observed surrounding both types of granules after 3 days of implantation. Additionally, microscopic examination of the specimens indicated considerable tissue regeneration and angiogenesis (Figs. 5 and 6). On day 3, both implants elicited a normal acute inflammation response with recruitment of inflammatory cells namely macrophages, lymphocytes, and rarely giant

cells. After 30 days of implantation no signs of chronic inflammation instead, in the vicinity of the material, high density connective tissue was observed with the presence of fibroblasts and blood vessels (Fig. 6).

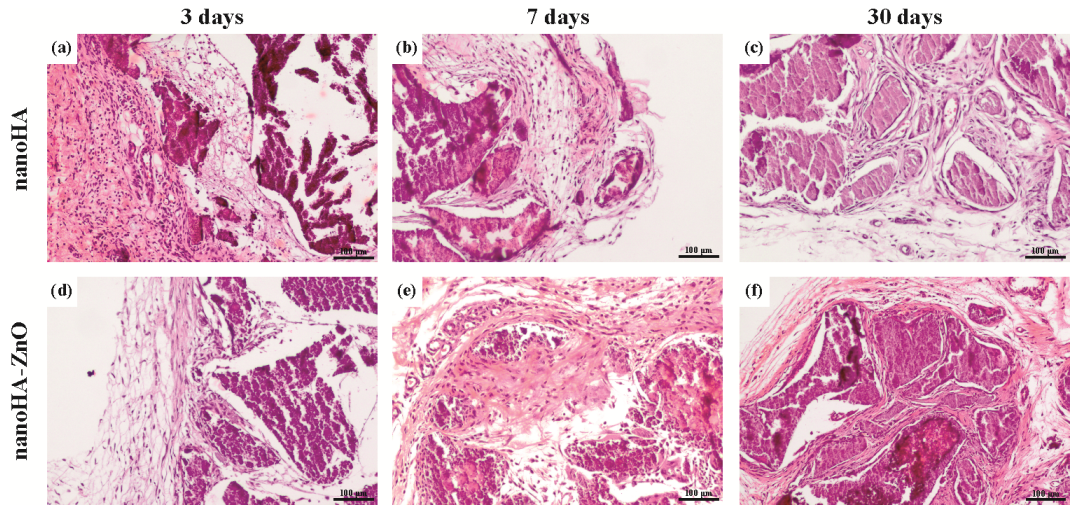

Figure 5: Histological analysis of HE stained sections of porous granules of pure nanoHA and nanoHA with 2% ZnO nanoparticles, after 3, 7 and 30 days of *in vivo* subcutaneous implantation. Scale bar 100 µm.

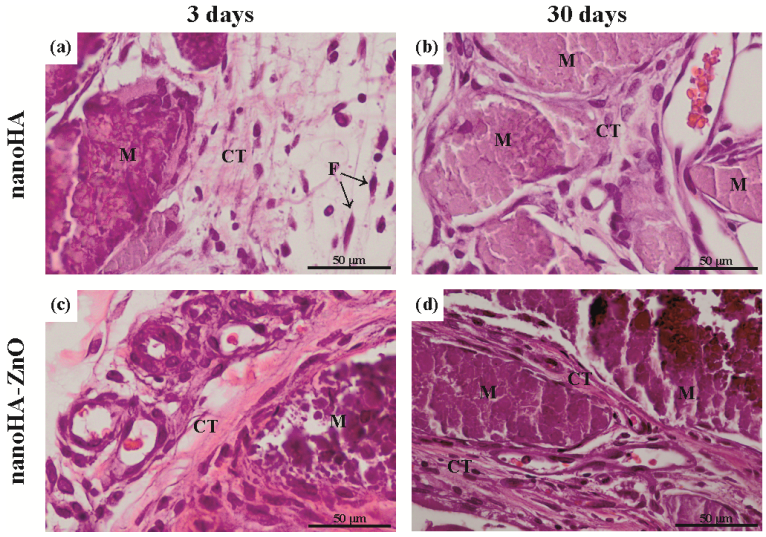

Figure 6: Histological analysis of HE stained sections of porous granules of pure nanoHA and nanoHA with 2% ZnO nanoparticles, after 3 and 30 days of *in vivo* subcutaneous implantation. M, material; F, fibroblasts; CT, connective tissue. Scale bar 50 µm.

To access the antibacterial effect of the composite *in vivo*, an inoculum of *S. aureus* was injected along the porous granules of pure nanoHA and nanoHA with 2% ZnO nanoparticles, and after 1 and 3 days the number of CFUs isolated from the homogenized samples (biomaterial and surrounding tissue) was quantified. As depicted in Figure 7, after 3 days of trial, the number of bacteria retrieved from the nanoHA-ZnO implants was significantly lower than that on pure nanoHA biopsies, for which a bacterial proliferation was observed between days 1 to day 3.

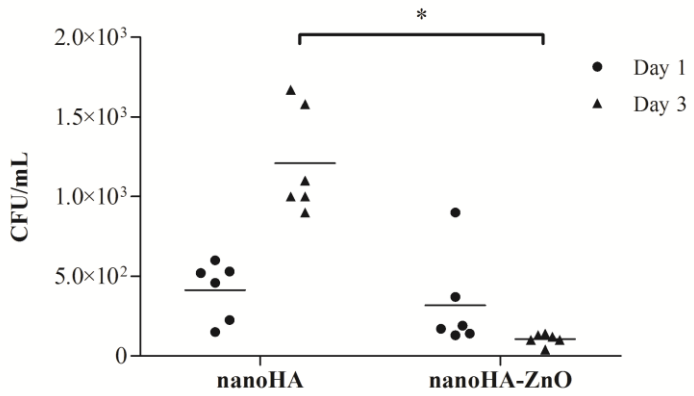

Figure 7: Number of viable *S. aureus* isolated from implanted porous granules of pure nanoHA and nanoHA with 2% ZnO nanoparticles, after 1 and 3 days of *in vivo* subcutaneous infection. *$p < 0.05$, significantly different.

4. DISCUSSION

The current goal for tissue engineering, in particular orthopaedic and dental research, is to produce high-quality bone substitute *in vitro*, capable to repair or replace bone tissue, that may be used as a clinical alternative to an autograft, the present 'gold standard' treatment [21]. However, the ultimate biomaterial should not only present high biocompatibility and bioactivity and appropriate mechanical properties but also antibacterial properties once biomaterial-related infections are a major threat to the current use of biomaterials. Infection seriously limits the healing and regenerative capacity of a tissue and remains a major limitation in the long-term utility of medical implants [22]. It is, therefore, crucial to identify an approach that might reduce bacterial growth without reducing mammalian cell functions.

Currently, several progresses have been made to use inorganic nanoparticles for biomedical applications. The advantage of using inorganic oxides is that they contain ions that are essential to humans and exhibit strong activity even when administered in small amounts [23]. Furthermore, they are much more stable and have a longer life than organic-based agents and, therefore, have superior safety and stability [11, 24]. Particularly, ZnO nanoparticles offer significant benefits for potential new nanotechnology-based biological applications, being already used in the cosmetic and sunscreen industry due to their transparency and ability to reflect, scatter, and absorb UV radiation, and as food additives [25]. Additionally, one of the most attractive features of these nanoparticles is their antibacterial activity, with several published reports confirming the efficacy of ZnO nanoparticle-based preparations as prophylactic agents against bacterial infections [26-28]. Thus, in order to provide antibacterial properties to a well-known biocompatible and bioactive ceramic nanoHA, which does not involve the use of antibacterial pharmaceutical agents, and with a clinically relevant morphology, three-dimensional nanostructured porous granules of HA incorporating small amounts of ZnO nanoparticles were produced.

In order to develop this type of biomaterial for bone tissue engineering it is important to meet certain criteria not only in terms of chemical composition, but also in terms of physical characteristics such as high and adequately distributed porosity. As depicted in figure 1, the three-dimensional porous granules synthesized by polymer replication method exhibited the required interconnected macro and microporosity throughout the scaffold. Microporosity (pore sizes below 10 μm), visualised in the pores walls, is important for cell adhesion while macroporosity (pore sizes higher than 100 μm) is essential for *in vivo* cell in-growth. In addition, the interconnected pore network allows better spatial organization for cell growth and extracellular matrix production, neovascularisation, higher levels of water and nutrients circulation as well as metabolic waste removal [1-3, 29]. Moreover, the granules nanofeatured surface produces a high surface area to volume ratio [18] and such morphological characteristic is known to increase selective protein and growth factors adsorption, promoting protein bioactivity, and improve subsequent tissue-forming cell functions leading to a better osteointegration *in vivo* [30-35]. Additionally, the observed nanoHA particles fusion obtained after the sintering treatment, is a very important feature because particles

detachment could cause significant problems, that is, free particles could migrate from the scaffold and induce an inflammatory response in the body; therefore, nanoparticles should never be released or rapidly dissolve [36]. Concerning ZnO nanoparticles, their high surface area, unique physiochemical properties, and improved surface reactivity [37] contribute to greater interactions with biological targets such as bacteria and host tissue [27, 38].

Several studies have shown that ZnO nanoparticles induce cytotoxicity in a cell-specific and proliferation-dependent manner, with rapidly dividing cells being more susceptible than normal quiescent cells. For instance, Taccola *et al* findings confirmed the ZnO nanoparticles selective cytotoxic action on rapidly proliferating cells, whether benign or malignant [39]. In other study, Premanathan *et al* demonstrated that human myeloblastic leukemia cells (HL60) were more susceptible to ZnO nanoparticles than the normal peripheral blood mononuclear cells (PBMCs) and Akhtar *et al* also observed that ZnO nanoparticles induce apoptosis in cancer cells (human hepatocellular carcinoma HepG2, human lung adenocarcinoma A549, and human bronchial epithelial BEAS-2B), while causing no impact on normal rat astrocytes and hepatocytes [23, 40]. In the present study, a similar profile was observed. The metabolic activity of human osteoblastic cancer cells (MG-63) exibhited a delay in cell proliferation after 3 days exposure to nanoHA-ZnO granules (Fig. 4) and the imaging flow cytometry analysis provided evidences that ZnO nanoparticles may induce apoptosis (Table I).

One of the primary mechanisms of nanoparticles toxicity, found for a diverse range of nanomaterials, is the free radical production. The sequential oxidation-reduction reactions may occur on the nanoparticle surface to produce reactive species, in a size-dependent way [37, 41]. Collectively, the aforementioned studies indicated that the underlying molecular mechanism of ZnO nanoparticles toxicity might involve the generation of reactive oxygen species (ROS), which is currently the best-developed paradigm for nanoparticle toxicity. The ROS production exceeds the cell's antioxidant capability resulting in oxidative stress, inflammation, and consequent irreparable damage to proteins, membranes, and DNA [37, 42]. Mitochondrial perturbation and DNA damage lead to the release of pro-apoptotic factors and, ultimately, programmed cell death. Sharma *et al* provides valuable insights into the mechanism of ZnO nanoparticles induced toxicity in HepG2 cell line [43]. However, mammalian cells are

able to limit toxicity and free radical damage due to a number of defences such as their antioxidant enzymes and their ability to phagocyte and dissolve nanoparticles, if free, by lysozomal fusion [37].

Similarly, the generation of ROS is also one mechanism proposed to explain their antimicrobial properties, enhanced by the greater specific surface area of nanoparticles [44]. The ROS production, like H_2O_2 or highly reactive hydroxyl radical and singlet oxygen, might trigger membrane lipid peroxidation and cause bacterial death [10]. As required, in the present study, a strong antibacterial effect against sessile bacterial growth was observed for granules of nanoHA with 2% ZnO (Fig. 3). Such effect is noteworthy once bacterial adhesion is the first and most important step of implant-related infection. Preventing bacterial adhesion could be a crucial way for its prevention [45]. Interestingly, at this wt %, ZnO only exhibited a low inhibition in cell growth with few apoptotic cells. Another potential mechanism of ZnO towards bacterial cells occurs through the leaching of zinc ions when the composite is suspended in an aqueous solution. The positively charged ions released into the medium can get attracted to the negatively charged cell membrane of bacteria by means of electrostatic interactions [46]. For instant, Seil *et al* observed a correlation between Zn ion released and antimicrobial activity [27]. However, in the present work, the measured concentration of zinc was found to be far lower than the minimum inhibitory concentrations identified by McCarthy *et al* or Zeelie *et al* against *S. aureus* (50 or 9 µg/mL respectively) [47, 48]. Such results may point out that ROS might be the major mechanism of nanoHA-ZnO granules antibacterial effect, which is in good agreement with other reports [10, 28]. Therefore, direct contact between ZnO particles and biological cells is not essential for enabling ZnO antibacterial activity [10]. The advantage of this strategy over others, with traditional antibiotics, relies precisely on the entrapment of ZnO nanoparticles within nanoHA substrate rather than as a coating, which enables a greater retention of antibacterial effects over time, avoiding the typical burst effect frequently detected for adsorbed drugs. Moreover, a strong effect was obtained using small amounts of ZnO nanoparticles, as compared to other studies [27].

In a clinical scenario, the increased susceptibility of an implant material to infections is partially due to impaired host defence at the implant site which can lead to biomaterial-adhering biofilms or, even worst, bacteria could infect surrounding tissue,

where they can reside intracellularly, becoming an important source for recurrent biomaterial-related infections [7]. Therefore, the *in vivo* study was designed to investigate the antibacterial efficacy as well as the biocompatibility of nanoHA granules with and without ZnO nanoparticles. The rodent subcutaneous model is widely accepted as the ideal initial model for investigating the biocompatibility of bone substitutes [7, 49, 50]. The 3 days, 1 and 4-week duration were selected because the intent was to assess the material during acute inflammation and after it had been largely resolved but before significant material degradation had occurred [49]. After 3 and 7 days implantation, both types of nanoHA granules activated the healing process with recruitment of inflammatory cells. This subsequently led to tissue formation around the material, particularly high density connective tissue morphology with the presence of fibroblasts and blood vessels [49]. Same newly formed eosinophilic connective tissues were also detected by Lee and collaborators in different bioceramics (HA with or without collagen) [35]. This rapid coverage of implant material by newly formed tissues may be an important additional event against bacterial infection. Concerning the antibacterial effect, 1 and 3-days of infection were selected once the first days after introducing an implant are crucial for the interactions of microorganisms with biomaterial and with the host immune system, which take place in the surroundings of the implants [51]. It is noteworthy that the *in vivo* biofilm prevention occurs despite the abundance of blood proteins that can potentially block the bactericidal action of ZnO nanoparticles and aid bacterial adhesion [52]. Taken together, nanoHA-ZnO porous granules had high antibacterial activity and did not induce any inflammation and toxicity *in vivo*.

5. CONCLUSIONS

Scaffold based tissue engineering has become a promising strategy in regenerative medicine. The experimental evidence presented herein supports the view that nanostructured porous granules of HA incorporated with different amounts of ZnO nanoparticles protect biomaterial surfaces from biofilm formation not only *in vitro* but also *in vivo*. Furthermore, the experimental strategy did not lead to emergence of resistant bacteria; it may mitigate concerns about multi-drug resistant super-bugs commonly seen in conjunction with approaches based on conventional antibiotics.

Finally, the three-dimensional and interconnected porous scaffolds supported tissue recovery, with the presence of new blood vessels and tissue regeneration *in vivo*. For these reasons, the porous granules of nanoHA-ZnO have the potential to improve numerous orthopaedic and dental applications, due to such antibacterial abilities while preserving biocompatibility, bioactivity and provision of adequate conditions for bone tissue regeneration.

ACKNOWLEDGMENTS

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CHAPTER IV

In vitro antimicrobial activity and biocompatibility of propolis containing nanohydroxyapatite

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ABSTRACT

The high number of biomaterial associated infections demands for new strategies to prevent this problem. In this study the suitability of nanohydroxyapatite (nanoHA)-based surfaces containing two Brazilian extracts of propolis (green or red ones) to prevent bacterial growth and biofilm formation, as well as its non-cytotoxic nature, was investigated. Optical density, colony forming units and MTT reduction assay were used to assess the materials' antibacterial activity against planktonic and sessile growth of *Staphylococcus aureus*. NanoHA matrix was able to absorb both types of propolis and the obtained results revealed the antibacterial effectiveness of the novel materials expressed as the reduction of bacterial growth and biofilm formation ability. Additionally, cell culture tests showed the growth of fibroblasts with high metabolic activity and without membrane damage. Therefore, these nanoHA-based surfaces containing natural products deriving from bees may be a promising bioactive biomaterial to be further studied with the aim of application to orthopaedic or dental devices.

Keywords: nanohydroxyapatite, propolis, antibacterial activity, cytocompatibility

1. INTRODUCTION

The application of medical devices either for temporary or permanent use has become an indispensible part of regenerative medicine. However, foreign bodies are associated with a substantial risk of bacterial and fungal infections and, in addition, the alarming phenomenon of loss of efficacy of traditional antibiotic therapies urges the discovery of new alternatives to solve and possibly even prevent this problem. Medical devices could generate niches for microbial adaptation capable to favouring emerging pathogens previously considered saprophytic species. Moreover, once a biofilm has formed, it can be very difficult to treat clinically because the microorganisms inside the biofilm are protected from phagocytosis and antibiotics. Solutions to avoid these adverse events could possibly rely on tuneable biomaterials surfaces [1-3].

Nanosized hydroxyapatite (HA) is the main inorganic component in natural bone, and synthetic HA has been widely used as a biocompatible and bioactive ceramic in many areas of medicine, but mainly for contact with bone tissue. Due to size effects and surface phenomena at the nanoscale, nanophase HA possesses unique properties when compared to its bulk-phase counterpart. The high surface-to-volume ratio, reactivity, and biomimetic morphologies make nanohydroxyapatite (nanoHA) more favourable in applications such as dental and orthopaedic implant coatings, to stimulate bone growth around the implant, leading to strong bone-implant binding, or as bone substitute filler [4, 5].

Regarding orthopaedic implants, the four most prevalent bacterial species, accounting for over 75% of infections, are *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Enterococcus faecalis,* while the remaining 25% consist of a list of over 50 species, among them bacteria of the *Enterobacteriaceae* family [6]. The microbial communities associated with peri-implantitis revealed higher levels of *Streptococcus* (*Streptococcus mutans* and non-mutans *Streptococcus*), *Butyrivibrio, Campylobacter, Peptococcus* and *Actinomyces* [1, 7].

In order to eliminate or substantially reduce the extent of bacterial attachment and biofilm formation on biomaterials surfaces, intensive efforts have been focused on the development of new surfaces, or on the improvement of the performance of existing antibacterial surfaces [8].

Due to the increasing bacterial resistance associated with chemotherapic agents, natural products are a promising source for the discovery of new pharmaceuticals. Among them, propolis (bee glue) is a natural resinous hive product produced by honeybees (*Apis mellifera*) from different plant sources. It is used by the bees to seal holes and cracks in their honeycombs, to smooth out the internal walls and to protect the colony from insect invasions and diseases [9, 10]. Propolis has been used in folk medicine for centuries in many regions of the world. Recently, propolis has been extensively used in the food industry as an additive for healthy food, beverages and nutritional supplements to improve health and prevent diseases. In fact, propolis exhibits a broad spectrum of biological properties, such as immunomodulatory, antitumoral, anti-inflammatory, antioxidant, and antimicrobial activities, among others [9, 11, 12]. The antibacterial and antifungal properties of propolis have been extensively investigated and, although its chemical composition is complex and linked to the phytogeographic origin, the activity of propolis has always been reported [13-20].

Under these circumstances, the aim of this work was to synthesize and characterize nanoHA-based surfaces containing two Brazilian ethanolic extracts of propolis (green and red ones). Thereafter, their antimicrobial properties, on planktonic and sessile growth phases of *S. aureus*, were evaluated as well as the materials' cytocompatibility on fibroblast cells.

2. MATERIAL AND METHODS

2.1. Bacterial strain and culture conditions

S. aureus ATCC 25923 was used in all experiments in this study. Stock cultures of microorganism were stored in brain heart infusion broth (BHI, HiMedia, India) supplemented with 20% glycerol at -80ºC. Working cultures were grown on BHI broth and agar (HiMedia, India) over 24 h at 37ºC.

2.2. Ethanolic extracts of propolis

Ethanolic extracts of red and green propolis (25%) were supplied by Pharma Néctar® (Belo Horizonte, Brazil). The botanic origin of red and green propolis is *Dalbergia ecastophyllum* and *Baccharis dracunculifolia*, respectively.

2.3. Synthesis of propolis containing nanoHA

NanoHA samples were prepared as previously described [21]. Briefly, nanoHA powder (nanoXIM·HAp202; Fluidinova S.A., Portugal) was pressed to cylindrical samples and heat-treated at 830ºC, with a 10 min plateau and applying a heating rate of 20ºC min^{-1} (Titan Platiniun 2000, EDG). For the following experiments, the samples were sterilized by dry heat (180ºC, 2 h).

In order to obtain propolis containing nanoHA, first the ethanolic extracts of red and green propolis were diluted in a hydroalcoholic solution (50% (v/v)) to prepare the solutions 6, 12 and 25 µg mL^{-1}. Then, the ceramic samples were immersed over-night, at room temperature, in these solutions. NanoHA samples were also immersed in a hydroalcoholic solution without propolis and were used as a control and further mentioned as non-treated nanoHA. After impregnation, the samples were dried at 37ºC for 2 h.

2.4. Physicochemical characterization of propolis containing nanoHA

2.4.1. Contact angle measurement

Surface hydrophobicity was evaluated using a contact angle measurement device (OCA 15, DataPhysics Instruments GmbH, Germany). The sessile drop technique was applied with ultrapure water at room temperature. Due to the absorbing nature of nanoHA surfaces, the drop deposition on material surface was recorded by a video charge-coupled device (CCD) camera and further analyzed to obtain the contact angle (SCA software, DataPhysics Instruments GmbH). Values reported are the average of five independent measurements.

2.4.2. Reversed-phase high performance liquid chromatography (RP-HPLC)

Samples of nanoHA impregnated with 25 µg mL^{-1} of propolis (red or green) were placed into 10 mL of methanol and sonicated for 1 h to release the propolis compounds from the ceramic matrix. This solution was analysed by RP-HPLC with a liquid chromatographer. The HPLC system consisted of a JASCO PU-2080 plus ternary pump, a manual injector equipped with a 20 µL sample loop and a JASCO MD-2015 plus diode array detector. A JASCO ChromPass Chromatography data system (version

1.8.6.1) allowed the control of the equipment and the data processing. The analytical column was a CC 250/4 Nucleosil 100-5 C18. The solutions were filtered with a 0.22 µm filter (Millipore) prior to 20 µL injected into the HPLC system. The column was eluted by using a linear gradient of solvent water (solvent A) and methanol (solvent B), starting with 30% of B (0-15 min) and increasing to 90% (15-75 min), held at 90% B (75-95 min), and decreasing to 30% of B (95-105 min) with a solvent flow rate of 1 mL min^{-1}. Chromatograms were recorded at 260 nm [15, 22]. The following authentic standards of phenolic acids and flavonoids were examined: ρ-coumaric, ferulic acid, cinnamic acid, gallic acid, quercetin, hesperidin and chrysin.

2.5. Antibacterial activity of propolis containing nanoHA

Antibacterial activity of propolis containing nanoHA against *S. aureus* growth was determined by quantifying the planktonic bacteria held in contact with the modified ceramic, by OD readings and CFU counts. Therefore, 1 mL of bacterial suspension in BHI broth (10^6 CFU mL^{-1}) was added to each well of a 24-well plate, containing the material samples. After 24 h incubation at 37ºC, the OD of bacterial suspensions (total number of bacteria) from each well was measured at 630 nm with a microplate reader. In parallel, an aliquot of these suspensions, from each well, was diluted and spread on BHI agar plates (viable bacteria). After incubation at 37ºC for 24 h, the number of bacterial colonies was counted and thereafter the CFUs were calculated. Non-treated nanoHA was used as control.

2.6. Biofilm formation on propolis containing nanoHA

The propolis containing nanoHA samples were also tested to evaluate their potential to prevent the sessile growth of *S. aureus* and biofilm formation. A bacterial suspension was prepared and adjusted to cell density of approximately 10^6 CFU mL^{-1} in BHI broth. Then, 1 mL of this bacterial suspension was inoculated onto material samples and after 24 h of incubation at 37ºC the bacterial suspension was removed and the samples were rinsed twice with 0.9% NaCl solution. Afterwards, three replicas were used to assess the metabolic activity of sessile bacteria by MTT assay while another three were used for visualization by confocal microscopy. For MTT reduction assay, 1

mL of 10% of (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide dye (MTT, Sigma-Aldrich) (5 mg mL^{-1} in phosphate-buffered saline) was added to each well, for 2 h at 37ºC. Following incubation, the MTT solution was removed from the wells and the material samples were transferred to new wells; 1 mL of dimethyl sulfoxide (DMSO, Synth, Brazil) was added for 15 min at room temperature. After mixing, the OD at 492 nm was measured using a microplate reader. A higher absorbance is related to a higher reduction of tetrazolium salt to purple formazan product, which indicates more viable *S. aureus* in the biomaterial surface. Results were expressed as a percentage of viable bacteria on non-treated nanoHA (0 µg ml^{-1}). For microscopic visualization (Spectral Confocal Microscope, Leica TCS SP5), the adherent cells were stained using a Live/Dead® BacLight Kit (Invitrogen, USA) with SYTO 9 and Propidium Iodide (PI), for 10 min at room temperature. The living cells appeared as green in colour (due to SYTO 9), while the dead cells were red (due to PI).

2.7. Cell culture

To access the material cytocompatibility, nanoHA impregnated with 12 µg mL^{-1} of red or green propolis was the selected material to be tested. Non-treated nanoHA was used as a control. The 3T3-L1 mouse fibroblast cells were seeded on material samples at a density of 10^5 cells mL^{-1} in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% bovine calf serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco). Cell cultures were incubated at 37ºC in a humidified atmosphere of 5% CO_2. After 1, 3 and 7 d of incubation, cell viability was evaluated through the MTT reduction assay and the lactate dehydrogenase (LDH) assay.

2.7.1. Metabolic activity

Fibroblast metabolic activity was evaluated using the MTT reduction assay. Briefly, after each culture time point, 10% of MTT solution (5 mg mL^{-1}) was added to each well and the plates were incubated for 3 h at 37ºC in a humidified atmosphere of 5% CO_2. Subsequently, the MTT solution was discarded and the materials transferred to new wells. The formed formazan crystals were dissolved with DMSO for 15 min at

room temperature, followed by absorbance measurement at 550 nm on a microplate reader (Multiskan Spectrum, Thermo Scientific).

2.7.2. Lactate dehydrogenase assay

The LDH activity was detected using an assay kit (Cyto Tox 96, Promega). Briefly, after every culture time point, the supernatants from each individual well were added to a 96-well plate along with LDH assay reagent (freshly prepared) and incubated for 45 min. Afterwards, HCl was added to stop the reaction and the absorbance values were recorded at 490 nm. Results were expressed as percentage of LDH release in control samples (non-treated HA).

2.8. Statistical analysis

All experiments were conducted in triplicate. The data are reported as mean ± standard deviation. The differences among groups were tested by one-way analysis of variance (ANOVA), followed by multiple comparisons among groups using the Tukey HSD *post hoc* test (IBM® SPSS® Statistics, versus 19.0). In all cases, $p < 0.05$ was considered to be statistically significant.

3. RESULTS

To evaluate the propolis potential on modified nanoHA surface properties, the contact angle measurements were performed (table 1). The hydrophilic behaviour was obtained for non-treated nanoHA (0 µg mL^{-1}) while, with the increase of propolis content on ceramic material, the surfaces exhibited increasing contact angles values, implying a decrease in hydrophilicity. This increase was more evident for nanoHA containing green propolis.

To confirm the chemical elements of propolis released from nanoHA substrate, the identification of flavonoid and other phenolic constituents was carried out by direct HPLC comparison with authentic standards and was based on retention time, co-chromatography, and the identity of absorption spectra. The results are shown in table 2. Both materials released coumaric acid, ferulic acid, hesperidin and quercetin. However,

cinnamic acid and chrysin were only detected for nanoHA-RP. Gallic acid was not detected for either of the materials.

Table 1. Contact angle values of nanoHA samples impregnated with different concentrations of red (RP) and green propolis (GP) solutions.

	Contact angle (º)	
	nanoHA-RP	nanoHA-GP
0 μg mL^{-1}	12.38 ± 1.15	12.38 ± 1.15
6 μg mL^{-1}	15.78 ± 2.62	32.66 ± 5.81[a]
12 μg mL^{-1}	19.69 ± 2.31	49.87 ± 2.89[a]
25 μg mL^{-1}	40.80 ± 6.97[a]	65.28 ± 6.13[a]

[a] $p < 0.05$, compared to non-treated nanoHA (0 μg mL^{-1})

Table 2. Phenolic acids and flavonoids released from nanoHA matrix impregnated with 25 μg mL^{-1} of red (RP) and green propolis (GP), detected by RP-HPLC (RT: retention time, min).

	RT (min)	
Compound	nanoHA-RP	nanoHA-GP
Gallic acid	nd	nd
Ferulic acid	2.37	2.37
ρ-Coumaric acid	2.52	2.53
Cinnamic acid	3.67	nd
Hesperidin	22.19	22.13
Quercetin	40.67	40.77
Chrysin	54.35	nd

nd: not detected

The effect of propolis containing nanoHA materials on the planktonic and sessile growth phases of *S. aureus*, after a 24 h period of exposure, is shown in figures (1-3). With regard to the planktonic bacteria, two methodologies, OD readings and CFU counts, were applied to evaluate the materials' bacteriostatic or bactericidal effect. In

comparison with non-treated nanoHA, all nanoHA samples containing either red or green propolis exhibited a significant reduction in the total number of bacteria as well as in the number of viable bacteria (figure 1). The nanoHA impregnated with the two highest concentrations of red propolis showed a remarkable reduction of 99% in the number of viable bacteria (figure 1(a)), while nanoHA with green propolis for the same concentrations showed a reduction of 45 and 61%, respectively (figure 1(b)). The effect of propolis on *S. aureus* cells appears to be a bactericidal one, with the red propolis having a stronger antibacterial activity in comparison to the green one.

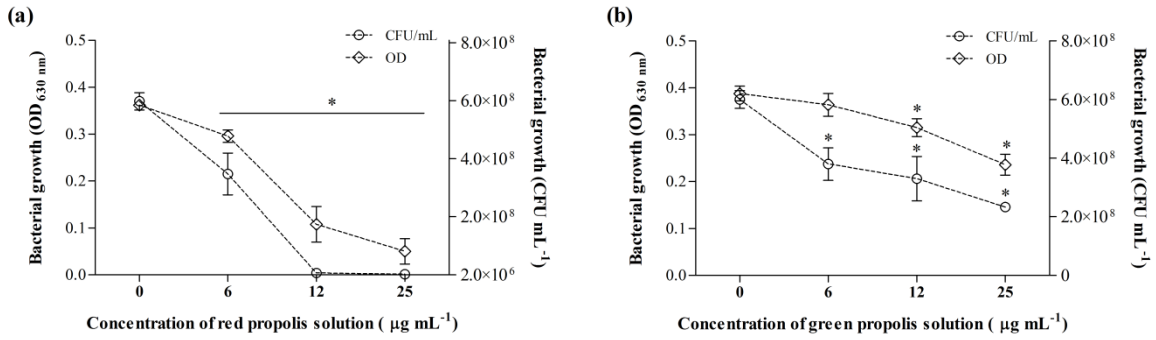

Figure 1. Planktonic growth of *S. aureus*, evaluated in terms of total cells (OD) and viable cells (CFU mL^{-1}), after 24 h incubation with nanoHA samples impregnated with different concentrations of red (a) and green (b) propolis solutions. $^{*}p < 0.05$, significant reduction compared to non-treated nanoHA (0 µg mL^{-1}).

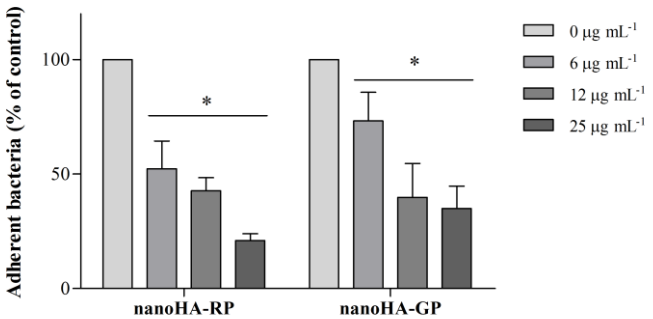

Figure 2. Sessile growth of *S. aureus* on nanoHA samples impregnated with different concentrations of red (RP) and green propolis (GP) solutions, as a percentage of viable bacteria on non-treated nanoHA (0 µg mL^{-1}), determined by MTT reduction assay, after 24 h of incubation. $^{*}p < 0.05$, significant reduction compared to non-treated nanoHA (0 µg mL^{-1}).

In order to investigate the effect of the propolis containing nanoHA samples on *S. aureus* biofilm formation, the metabolic activity of the bacterial cells attached on the material surface was evaluated by MTT reduction assay and further assessed by confocal microscopy using a Live/Dead staining. The results obtained showed a general reduction of *S. aureus* activity in a concentration-dependent way which was significant at just 6 µg mL^{-1} for both tested propolis types, as depicted in figure 2. Moreover, the nanoHA impregnated with the highest concentration of red propolis solution was able to inhibit 80% of the staphylococcal biofilm. Lastly, the ability of propolis containing nanoHA to prevent bacterial sessile growth was confirmed by fluorescence microscopy (figure 3). After 24 h of culture, a high number of viable *S. aureus* on pure nanoHA samples was observed. Comparatively, propolis containing nanoHA exhibited an overall decrease of bacterial density in a concentration-dependent way, followed by an increase in dead cells.

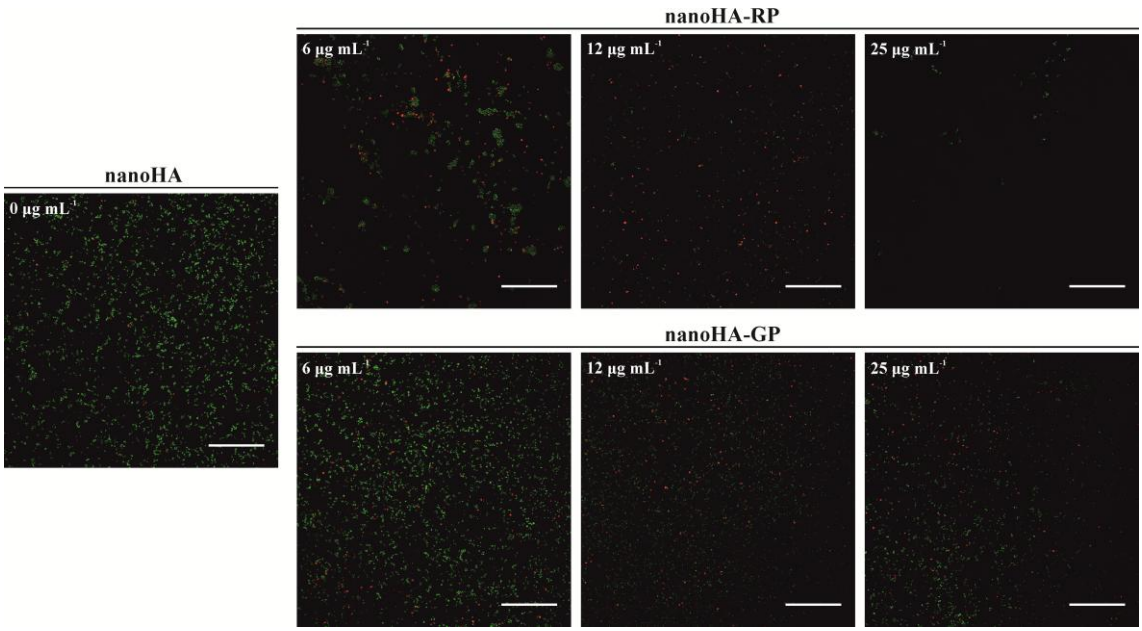

Figure 3. Fluorescence images showing the viability of *S. aureus* on nanoHA samples impregnated with different concentrations of red (RP) and green propolis (GP) solutions. The living bacteria are coloured green, while the dead ones are coloured red. Scale bar 50 µm.

In general, all nanoHA samples impregnated with red or green propolis affected the growth of planktonic cells as well as sessile bacteria in a similar way.

The potential application of nanoHA supplemented with red or green propolis in biomedical devices has been evaluated. For that purpose, the material cytocompatibility was analyzed using fibroblastic cell cultures, by MTT reduction assay and LDH release, after 1, 3 and 7 d of culture. NanoHA impregnated with a solution of 12 µg mL^{-1} of red or green propolis was selected once it was at the concentration that proved able to promote a reduction of around 50% in the number of sessile bacteria on material surface. For nanoHA with red propolis, for all time points, a high metabolic activity of fibroblast cells was observed, as indicated by the increase in MTT reduction, and comparatively to non-treated nanoHA (figure 4). Similar results were obtained for nanoHA with green propolis, except for day 1, where a fraction of just 57% remained active. Nevertheless, at day 3 and 7, the metabolic activity reached similar values to those of non-treated nanoHA. Material cytotoxicity was evaluated by LDH assay. LDH is a stable cytosolic enzyme released upon membrane damage in necrotic cells [23]. The analysis of LDH release levels at different time points in the culture medium did not reveal significant changes between cells cultured on treated and non-treated nanoHA, as depicted in figure 5. This means that the materials induced no, or only marginal, cytotoxicity on fibroblastic cells.

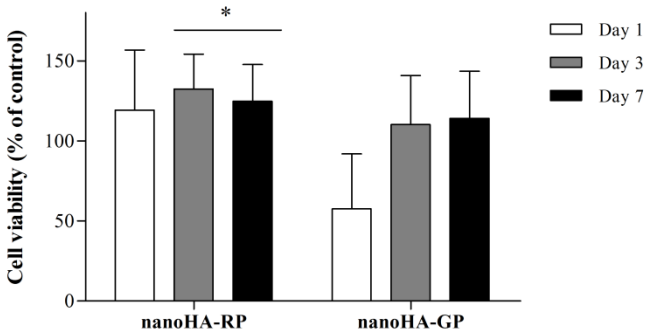

Figure 4. Metabolic activity of fibroblast cells cultured on nanoHA impregnated with red (RP) and green propolis (GP) for 1, 3 and 7 d, estimated by MTT assay. Non-treated nanoHA was considered 100% of cell viability. $^{*}p < 0.05$, compared to non-treated nanoHA (0 µg mL^{-1}).

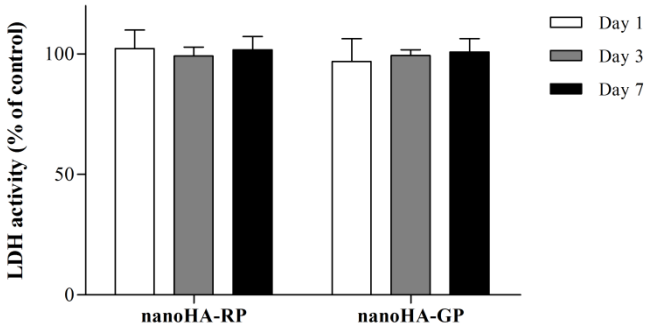

Figure 5. Cytotoxicity of nanoHA impregnated with red (RP) and green propolis (GP) to fibroblast cells, after 1, 3 and 7 d of culture, evaluated by LDH assay. Results were expressed as a percentage of LDH release in control samples (non-treated HA).

4. DISCUSSION

Propolis comprises a broad spectrum of biological properties and, as a result, it has been explored as a novel agent for biomedical applications [24-28]. Nevertheless, the use of propolis containing nanoHA as a bioactive biomaterial, which is useful in orthopaedics or dental applications to stimulate bone growth and to prevent biomaterial-associated infections, has never been tested. Therefore, the present study evaluates the effect of these new materials based on nanoHA impregnated with propolis extracts on *S. aureus* growth, a major pathogen related to implanted medical devices [1, 6], as well as on the cytocompatibility with fibroblastic cells. Ethanolic extracts of two distinct types of propolis were used, namely Brazilian red propolis derived mainly from the surface of *D. ecastophyllum*, located in mangrove areas in the north-eastern Brazil [29, 30]; and Brazilian green propolis or alecrim propolis whose botanical origin is mainly *B. dracunculifolia* exudates, the dominant source of propolis in south-eastern Brazil [22, 31].

The nanoHA-based surfaces containing red or green propolis exhibited a significant inhibition of *S. aureus* planktonic and sessile growth (figures 1 and 2). Wherein, the nanoHA containing red propolis displayed a higher antibacterial activity when compared to the absorbing green counterpart, as was also confirmed by confocal microscopy (figure 3). A large number of constituents have been identified in Brazilian propolis samples and the antibacterial activity has been mainly attributed to phenolic

compounds, especially flavonoids, phenolic acids and esters [32-34]. However, the higher antibacterial activity expressed by red propolis may be attributed to the difference in its chemical composition and concentration of components which derives mainly from isoflavones (daidzein, formononetin and biochanin A), and its total phenolic compounds content which is higher than any ever found on other Brazilian propolis and which seems to contribute to higher antibacterial effect [19, 29, 35, 36]. In contrast, the antibacterial activity of green propolis derives mainly of flavonoids, aromatic acids, and esters present in resins, galangin, kaempferol, pinostrobin, and pinocembrin which are known to be more effective agents against bacteria. Ferulic acid and caffeic acid also contribute to the bactericidal action of propolis [12, 32, 37]. Salomão *et al* [20] found a positive correlation between the ρ-coumaric acid amount in propolis and the bactericidal effect on *S. aureus*. Some of these compounds were identified in the present work by RP-HPLC analysis (table 2), including ρ-coumaric acid. Nevertheless, the observed differences in the chemical profile could provide an explanation for the differences found in the antibacterial activities of the distinctive types of propolis. Moreover, the compound's concentration may also dictate the observed effects. Cinnamic acid and chrysin are typical constituents of propolis. Their absence in the solution from green propolis containing nanoHA may be related to their presence below the detection limits [22]. Additionally, propolis compound's affinity to nanoHA substrate may have dictated the chemical and antibacterial profiles. As highlighted by the contact angle measurements (table 1), an increase in such values was observed which is related to the lipophilic nature of propolis [12, 38]. However, this increase was not as high for nanoHA containing red propolis which may be due to its chemical profile consisting of substances of a more polar nature [36], that in turn may have a higher affinity for the hydrophilic nanoHA substrate [21]. The higher antibacterial activity against planktonic growth may be attributed to the diffusion of active constituents of propolis towards the ceramic matrix.

As described, the composition of propolis is very complex and its antibacterial activity is linked to some of the above mentioned constituents or to the synergistic action of several such components. Some mechanisms of propolis activity on the bacterial growth have been reported, such as inhibition of protein synthesis or functional and structural damage on the bacterial cell membranes and cell walls [39, 40]. Propolis

phenolics are generally linked to destabilization and permeabilization of the cytoplasmatic membrane, protein denaturation or inhibition of extracellular enzymes [41, 42]. Galangin, caffeic and cinnamic acids are enzymatic inhibitors responsible for blocking bacterial growth and proliferation. Other flavonoids, such as quercetin, affect bacterial membrane potential, causing increased membrane permeability, and inhibit bacterial motility [43-45]. It is clear that the mechanism of propolis activity on bacterial cells is complex and a simple analogy cannot be established with the mode of action of any classic antibiotics. Interestingly enough, synergy between propolis and a range of antibiotics has been shown against some bacteria, and when using a mixture of both products the effective dose of antibiotics was reduced [40, 43, 46-48].

In addition, propolis may also suppress microbial virulence factors. Scazzocchio *et al* [46] demonstrated a multiple action of propolis against different virulence factors of some Gram-positive bacteria of clinical interest. *Staphylococcus*'s virulence factor coagulase was completely suppressed by propolis as lipase, and a dose-dependent prevention of biofilm formation was reported. Mirzoeva *et al* [43] found that the anti-motility action of propolis components may play an important role in inhibition of bacterial pathogenesis and the development of infection once bacterial motility is important in virulence, as it guides bacteria to their sites of adherence and invasion.

Besides their antibacterial activity, the results obtained with fibroblasts cultures are noteworthy as they revealed that nanoHA impregnated with propolis was non-cytotoxic. Instead, nanoHA impregnated with red propolis led to an increase in cell metabolic activity in comparison with non-treated nanoHA (figure 4). The LDH assay also confirmed these results once that similar profiles of LDH release were observed for treated and non-treated nanoHA (figure 5). These results are in agreement with other studies, using fibroblasts or other cell types [23, 45, 49, 50]. Al-Shaher *et al.* [51] reported that propolis exerts minimal toxicity on both fibroblasts of the periodontal ligament (PDL) and dental pulp at concentrations of 4 mg mL^{-1} or lower. Likewise, Gjertsen *et al.* [52] observed that propolis not only decreased apoptosis but also increased the metabolic activity and proliferation of PDL cells.

Our data, together with the widespread appearance of antibiotic resistance and propolis large broad-spectrum antimicrobial activity and its lower risk for the development of resistance, may indicate that propolis can be, by itself, a bioactive

product, or be a promising source of new bioactive compounds to prevent and treat biomaterial-associated infections.

5. CONCLUSION

The potential pharmacological activity of natural products, especially antimicrobial activity, has attracted the attention of several researchers. The results obtained in this work indicated that propolis containing nanoHA was able to hinder the bacterial growth and biofilm formation of *S. aureus*, as well as be non-cytotoxic to fibroblast cells. These findings encourage the future studies of these materials in terms of application to dental and orthopaedic implant coatings, to stimulate bone growth around the implant, and to prevent implant associated infections.

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CHAPTER V

Inhibitory effect of 5-aminoimidazole-4-carboxamidrazones derivatives on *Candida* spp biofilm on nanohydroxyapatite substrate

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ABSTRACT

Candida can adhere and form biofilms on biomaterials commonly used in medical devices and is a key attribute that enhances its ability to cause infections in humans. Furthermore, biomaterial-related infections represent a major therapeutic challenge since *Candida* biofilms present considerable resistance to most conventional antifungal therapies. As a result, there is a strong medical but also economical motivation for the development of newer and more potent antifungal agents. In the present work, the activity of three novel imidazole derivatives, 5-aminoimidazole-4-carboxamidrazones, namely 2h, 2k and 2l, to prevent *Candida albicans* and *Candida krusei* biofilm formation, as well as to eradicate pre-formed *Candida* spp biofilms on nanohydroxyapatite (nanoHA) substrates were investigated. To address these goals, both quantitative methods by cultivable cell numbers and qualitative evaluation by scanning electron microscopy were used. Compounds cytocompatibility towards osteoblast-like cells was also evaluated after 24 h and 48 h of exposure, through resazurin assay. The three tested antifungal agents displayed strong inhibitory effect on biofilm development by both *Candida* species as potent *in vitro* activity against sessile *C. albicans* cells. Additionally, morphological changes following treatment with the imidazole derivatives were observed by SEM on nanoHA surfaces for both *Candida* species. Regarding cytocompatibility, a time- and concentration-dependent effect was observed. Together these findings indicated that the potent activity of 5-aminoimidazole-4-carboxamidrazones derivatives on *Candida* spp biofilms, in particular 2l compound, is worth further investigating, aiming at developing preventive approaches to fight biofilm-associated infections involving biomaterials.

Keywords: 5-aminoimidazole-4-carboxamidrazones, *Candida* biofilm, nanohydroxyapatite, cytocompatibility

1. INTRODUCTION

Yeast cells have the ability to adhere and colonize surfaces of a wide range of synthetic materials of polymeric, ceramic, and metallic compositions, often used in medical devices, in almost the same manner as to host mucosal surfaces [1-4]. In particular, *Candida* species are commensal microorganisms that colonize skin and mucosal surfaces in healthy humans thus facilitating their contact with most implanted biomaterials [5, 6]. Accordingly, the increase in fungal infections in the last decades has almost paralleled the increase and widespread use of a broad range of indwelling medical devices, mainly in populations with impaired host defences [5]. Although these infections are considered rare, little is known about their prevalence, the accuracy of the currently used diagnostic protocols, or the most effective treatment. As a result, they pose a serious health problem in the future. The vast majority of those infections is caused by *Candida* species. Particularly *C. albicans* shows the highest virulence among all the currently known medically relevant species of *Candida* and is the most common fungal species associated with biofilm formation in the clinical setting [7-9]. Nonetheless, other non-*albicans* species as *Candida glabrata*, *Candida parapsilosis*, *Candida krusei* and *Candida tropicalis* have been implicated in biofilm-related infections and are increasingly recognized as nosocomial pathogens of clinical interest [5, 9-11]. The most important virulence trait for numerous *Candida* species in the pathogenesis of device-related infections is the formation and establishment of proliferating biofilms [5, 12]. These are well structured microbial communities where the cells bind tightly to a surface and become embedded within a protective self-produced extracellular polymeric matrix [5]. The cellular communities formed on biomaterial surface have a characteristic architecture and phenotypic features that are distinct from their planktonic, free-floating counterparts [2, 13, 14]. Mature *C. albicans* biofilms have a complex three-dimensional architecture with wide spatial heterogeneity, consisting of a mixture of morphological forms namely yeast, pseudohyphae and hyphae, encased within a matrix of polysaccharides, carbohydrates and proteins [15, 16]. It has been shown that *C. albicans* produces larger and more complex biofilms than other *Candida* species [17]. *Candida* colonization of medical devices not only precedes infection, but it can also adversely affect the performance of the implanted medical device [5]. Once *Candida* biofilms are formed *in vivo*, removal of the substrate that is

supporting the biofilm growth, together with long term antifungal therapy administration, are almost always required to control the infection and to avoid potentially fatal consequences, since yeast detachment from biofilm can cause an acute fungemia and/or life-threatening systemic infections [2, 12, 18]. Unfortunately, often removal is impossible, due to the patient's condition, the anatomic location, or underlying disease. From the clinical perspective, the most important features of *Candida* biofilms are their ability to withstand host defence mechanisms and their increased resistance to conventional antifungal agents, often resulting in serious and persistent infections [1, 13]. Together with the emergence of *Candida* species resistant to azole drugs [10, 19], there is a continuous need for the development of newer and more potent antifungal agents that are also effective against *Candida* biofilm infections. In a previous work, the synthesis and antifungal activity of a series of novel 5-aminoimidazole-4-carboxamidrazones were reported by our group [20]. These new imidazole derivatives exhibited promising antifungal activity and three of them, (*Z*)-5-amino-*N*'-aryl-1-methyl-1*H*-imidazole-4-carbohydrazonamides 1 [aryl=phenyl (2h), 4-fluorophenyl (2k), 3-fluorophenyl (2l)], have proven to be very effective against different clinically important yeasts, such as *C. albicans*, *C. krusei*, *C. parapsilosis* and *Cryptococcus neoformans* [20]. In this context, the aims of this study were: (i) to evaluate the effectiveness of these three novel antifungal agents to impair *C. albicans* and *C. krusei* biofilm formation on nanohydroxyapatite substrates, which is a well-known biocompatible and bioactive calcium phosphate ceramic material; (ii) to determine the compounds interference with a pre-formed *Candida* spp biofilm; and (iii) to assess the compounds cytocompatibility towards osteoblast-like cells (MG-63 cell line).

2. MATERIALS AND METHODS

2.1. Preparation of nanohydroxyapatite samples

Nanohydroxyapatite (nanoHA; nanoXIM·HAp202; Fluidinova S.A.) powders, uniaxially pressed as cylindrical disc samples, were sintered at 830ºC during 15 min using a heating rate of 20ºC/min, as described elsewhere [21, 22]. Prior to biological experiments, the biomaterials were sterilized by dry heat (180ºC, 2 h).

2.2. Antifungal agents

The stock solutions of 5-aminoimidazole-4-carboxamidrazones derivatives, namely 2h, 2k and 2l, were prepared as described elsewhere [20] and were freshly solubilised in dimethyl sulfoxide (DMSO, Applichem) at 10 mg/mL. Subsequent dilutions were made in yeast nitrogen base (YNB, Fluka) supplemented with glucose to obtain the desired concentrations, just prior the assays.

2.3. *Candida* strains and growth conditions

Candida albicans and *Candida krusei* reference ATCC strains 10231 and 6258, respectively, were subcultured and grown in Sabouraud dextrose agar plates (SDA, Liofilchem) at 37ºC for 24 h. After cultured, cells were harvested and diluted to the desired concentrations with glucose supplemented YNB medium.

2.4. Effect of 5-aminoimidazole-4-carboxamidrazones derivatives on *Candida* spp biofilm formation

The compounds potential to prevent biofilm formation of *C. albicans* and *C. krusei* on nanoHA surface was evaluated using an adapted method described by [23]. Briefly, 50 μL of yeast suspension in YNB medium (final concentration of 5×10^2 or 1×10^6 cells/mL) were added to equal volume of compounds solutions (1:2 dilutions) into the wells of a flat bottom 96-well plate, containing the nanoHA discs. The yeast cells were allowed to adhere on discs surface for 24 h at 37ºC with moderate shaking. Discs of nanoHA incubated with yeast suspension and YNB medium were used as control (drug-free). After incubation the medium was removed and the biomaterials were gently rinsed twice with saline solution (0.9% NaCl, JT Baker) to remove non-adherent cells. The anti-biofilm effect was determined by colony-forming units (CFU) and scanning electron microscopy (SEM), as described below.

2.5. Effect of 5-aminoimidazole-4-carboxamidrazones derivatives on pre-formed *Candida* spp biofilm

The ability of imidazole derivatives to eradicate established *C. albicans* and *C. krusei* biofilms were assessed using an adapted method described herein [24]. Briefly,

100 μL of standardized yeast suspension in YNB medium (final concentration of 5×10^2 or 1×10^6 cells/mL) were added into the wells of a flat bottom 96-well plate, containing the nanoHA discs. The plates were kept at 37ºC for 24 h, with moderate shaking, to allow biofilm formation on nanoHA surface. After incubation, the medium was discarded and each biomaterial sample harbouring biofilm was rinsed once with saline solution to eliminate non-adherent cells. With sterile forceps the nanoHA discs were transferred to a new microtitre plate. The compounds (100 μL per well), at different concentrations, were added into the wells and the plates were incubated for an additional 24 h period at 37ºC, under moderate shaking. As control, *Candida* cells were incubated with YNB medium (drug-free). Following incubation, the medium was removed and the biomaterials were gently rinsed twice with saline solution. The antifungal effect of 5-aminoimidazole-4-carboxamidrazones derivatives against sessile cells was assessed by CFU and SEM, as described below.

2.6. *Candida* viability assay

The number of cultivable yeast cells, adhered on biomaterial surface, was determined by CFU enumeration. Briefly, nanoHA discs were transferred to an Eppendorf containing 1 mL of saline solution and were sonicated for 5 min in an ultrasonic bath (35 kHz, Bandelin-RK 156). The solutions containing the dispersed cells were then appropriately diluted and spread on SDA plates. After inoculation, the plates were incubated at 37ºC for 24 h. The number of colonies were counted and expressed as CFU/mm^2.

2.7. Scanning electron microscopy

The morphological effect of 5-aminoimidazole-4-carboxamidrazones on *C. albicans* and *C. krusei* cells was observed by SEM. Initially, the cells on nanoHA surface were fixed with 3% (v/v) glutaraldehyde solution in cacodylate buffer (Sigma-Aldrich) for 30 min at room temperature. Subsequently, the samples were dehydrated in a series of ethanol-water solutions followed by a series of hexamethyldisilazane (HMDS, Sigma-Aldrich)-ethanol solutions with concentrations ranging between 50% and 100%, for 10 min each, and finally air dried overnight. The samples were attached

on aluminium stubs with carbon tape, sputter-coated (SPI-Module) with a thin conductive film of Au-Pd alloy and then imaged by SEM using a FEI Quanta 400 FEG/ESEM microscope, under 500x and 5000x magnification in high-vacuum mode, at 15 kV accelerating voltage.

2.8. Cytocompatibility of 5-aminoimidazole-4-carboxamidrazones derivatives

Osteoblast-like cells (MG-63 cell line) were cultured in alpha minimum essential medium (α-MEM, Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco), 1% (v/v) penicillin-streptomycin (100 IU/mL penicillin and 2.5 µg/mL streptomycin, Gibco), and 1% amphotericin B (2.5 µg/mL, Gibco), at 37ºC, in a humidified atmosphere of 5% CO_2 and 95% air. The compounds solutions were diluted in supplemented α-MEM to appropriate concentrations that comprised all values previously assessed. Similarly to what was done for the microbiological studies, two approaches were tested: (a) 50 µL cell suspension (final concentration 10^4 cells/mL) was simultaneously seeded with equal volume of freshly prepared compounds solution, into 96-well plates containing the nanoHA discs. The plates were then incubated for 24 and 48 h; (b) cells (100 µL per well at 10^4 cells/mL) were allowed to attach on nanoHA surface for 24 h prior exposure to freshly prepared compounds dilutions (100 µL per well) for additional 24 and 48 h incubation time. For both approaches, compounds-free culture medium was used as a negative control. After the incubation periods, the cellular metabolic activity was determined by rezasurin assay. Thus, cells were incubated with resazurin solution (0.1 mg/mL, Sigma-Aldrich) for 3 hours at 37ºC. Afterwards, the supernatant had the fluorescence intensity measured in a fluorimeter (excitation: 530 nm and emission: 590 nm, Synergy HT, BioTek).

2.9. Statistical analysis

Each condition was tested in triplicate and two independent experiments were performed. The experimental data were expressed as the mean ± standard deviation and analyzed using IBM® SPSS® Statistics (vs. 20.0, SPSS, USA). One-way analysis of

variance (ANOVA) followed by the *post hoc* Tukey HSD multiple comparison test, which was used to denote the significance level ($p < 0.05$).

3. RESULTS

3.1. Ability of 5-aminoimidazole-4-carboxamidrazones derivatives to prevent *Candida* spp biofilm formation

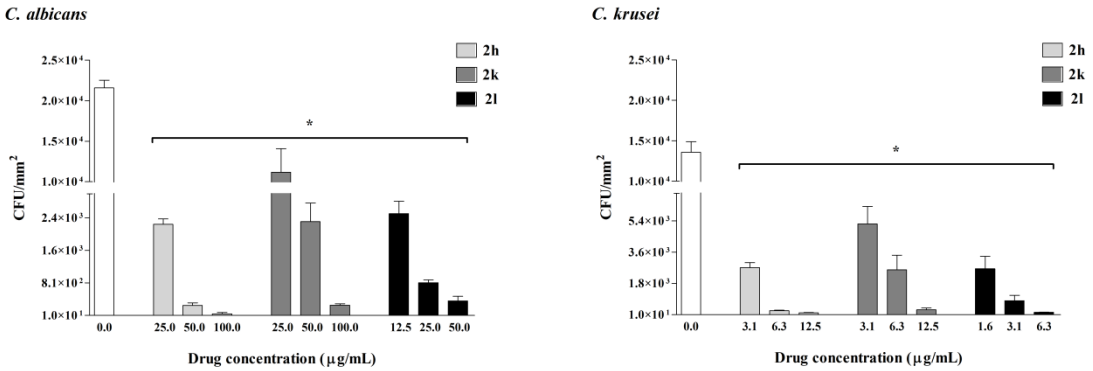

Figure 1. *In vitro* effect of 5-aminoimidazole-4-carboxamidrazones derivatives against *C. albicans* and *C. krusei* biofilm growth on nanoHA surface. Yeast cells were allowed to adhere in the presence of different concentrations of antifungal agents and after 24 h incubation the number of cultivable yeast cells was determined by CFU enumeration (CFU/mm^2). (*) indicates significant difference between antifungal treatment and the control (drug-free) ($p < 0.05$).

Based on a previous work by Ribeiro *et al* [20] where the minimal inhibitory concentration (MIC) of 2h, 2k and 2l compounds was determined against *C. albicans* and *C. Krusei*, the concentrations tested in the current study were thereafter established and they are the MIC, 2-fold superior or 2-fold inferior to MIC, for each *Candida* species.

Initially, yeast cells were allowed to adhere to nanoHA surface in the presence of antifungal agents and the effect of these different concentrations on biofilm growth of *C. albicans* and *C. Krusei* were assessed using two distinct initial inoculums. For an initial inoculum of 10^6 CFU/mL, the three imidazole derivatives were able to significantly inhibit the adhesion of both *Candida* species, for all tested concentrations, compared to that on drug-free (control) biomaterials (Fig. 1). In particular, the two

highest concentrations tested showed a reduction higher than 80% for both *Candida* species. As foreseen, using a lower inoculum (5×10^2 CFU/mL), the three antifungal agents enabled a total inhibition of *C. albicans* and *C. krusei* adhesion on nanoHA surface, for the tested concentrations (data not shown).

3.2. Effect of 5-aminoimidazole-4-carboxamidrazones derivatives on pre-formed *Candida* spp biofilm

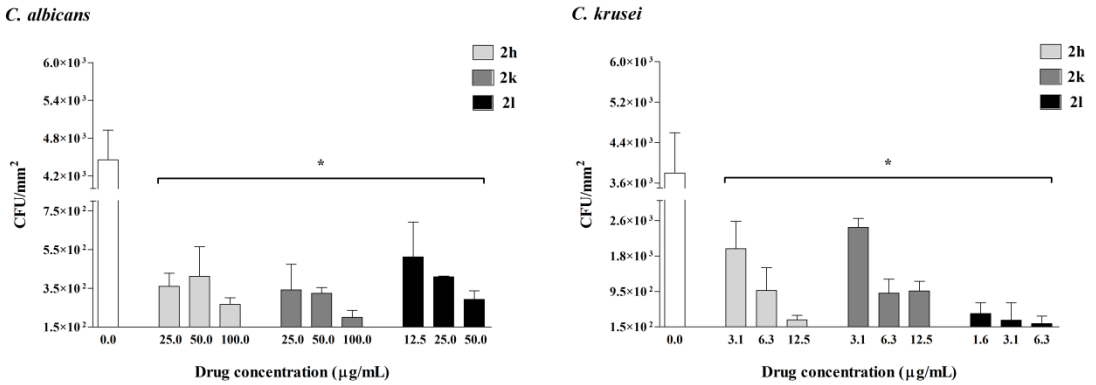

Figure 2. Number of remaining viable cells on nanoHA surface after treatment of 24 h-biofilm of *C. albicans* and *C. krusei* with different concentrations of 5-aminoimidazole-4-carboxamidrazones derivatives, expressed as CFU/mm^2. Initial yeast inoculum of 5×10^2 CFU/mL. *$p < 0.05$, differences statistically significant compared to control (drug-free).

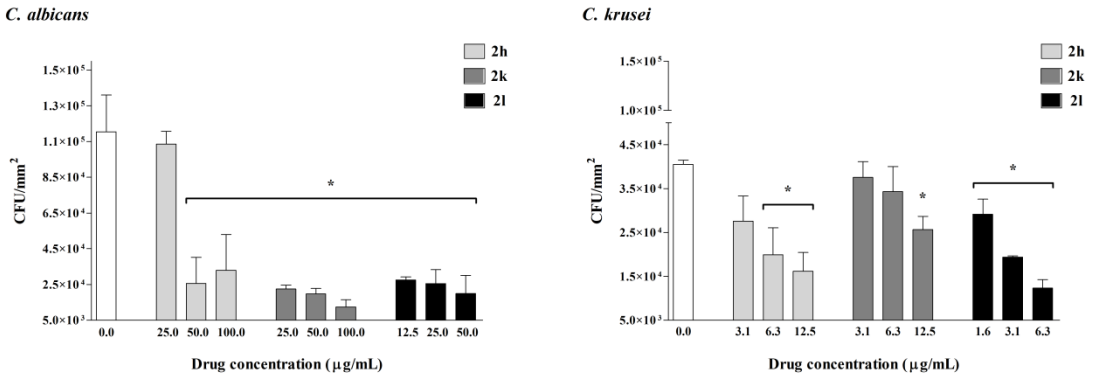

Figure 3. Number of remaining viable cells on nanoHA surface after treatment of 24 h-biofilm of *C. albicans* and *C. krusei* with different concentrations of 5-aminoimidazole-

4-carboxamidrazones derivatives, expressed as CFU/mm^2. Initial yeast inoculum of 10^6 CFU/mL. *$p < 0.05$, differences statistically significant compared to control (drug-free).

To explore the efficacy of imidazole derivatives in developing biofilms, *C. albicans* and *C. krusei* were cultured, at two different initial inoculums, for 24 h on nanoHA discs to allow yeast cells attachment. The pre-formed biofilms were then exposed to the antifungal agents and after 24 h, the number of viable adherent cells was quantified by cultivable cell number assay (Figs. 2 and 3). As depicted in Figure 2, using an initial inoculum of 5×10^2 CFU/mL, a significant reduction (> 90%) in the number of remaining viable *C. albicans* cells was achieved, when compared to non-treated (drug-free) biofilms, for all assessed conditions. Regarding *C. krusei*, 2l compound exhibited a sharp reduction of 88% for concentration as low as 1.6 μg/mL, whereas for 2h and 2k compounds a concentration-dependent effect was observed. Nevertheless, for the three tested concentrations a significant reduction in *C. krusei* sessile cell number was observed.

A second set of experiments was performed with a higher initial inoculum (10^6 CFU/mL) (Fig. 3) and despite this increase, the three imidazole derivatives displayed similar effect for the two highest concentrations against *C. albicans* sessile population when compared to the previously indicated results (Fig. 2). On the other hand, for *C. krusei* a concentration-dependent effect was noticed. Even so, the observed reduction never reached values higher than 61%, 38% and 70% for the highest concentration tested of 2h, 2k and 2l compounds, respectively (Fig. 3).

3.3. Direct visualization of 5-aminoimidazole-4-carboxamidrazones derivatives effect on *Candida* spp biofilm

SEM analysis, as exemplified in Figure 4, had shown a reduction in the number of adherent cells on nanoHA surface for both C*andida* species, after incubation with the three components tested, either for biofilm prevention or biofilm elimination experimental approaches. Additionally, *C. albicans* exhibited a highly heterogeneous structure, with morphological switch between yeast, pseudohyphae and hyphae, after exposure to the compounds, more evident for 2h compound, while on drug-free samples a predominately yeast morphology was observed (Figs. 4 and 5). Higher magnification

images also enabled the visualization of round or oval blastospores (yeast forms) aggregated either in pairs or clumps for both *Candida* species with some budding yeasts (Figs. 5 and 6). However, while *C. albicans* structures had a full shape and smooth surface (Fig. 5), some *C. krusei* cells treated with 5-aminoimidazole-4-carboxamidrazones derivatives appeared shrunk, wrinkled and disc-like depressions, with different depths, were present at the cell surface, as indicated in Figure 6.

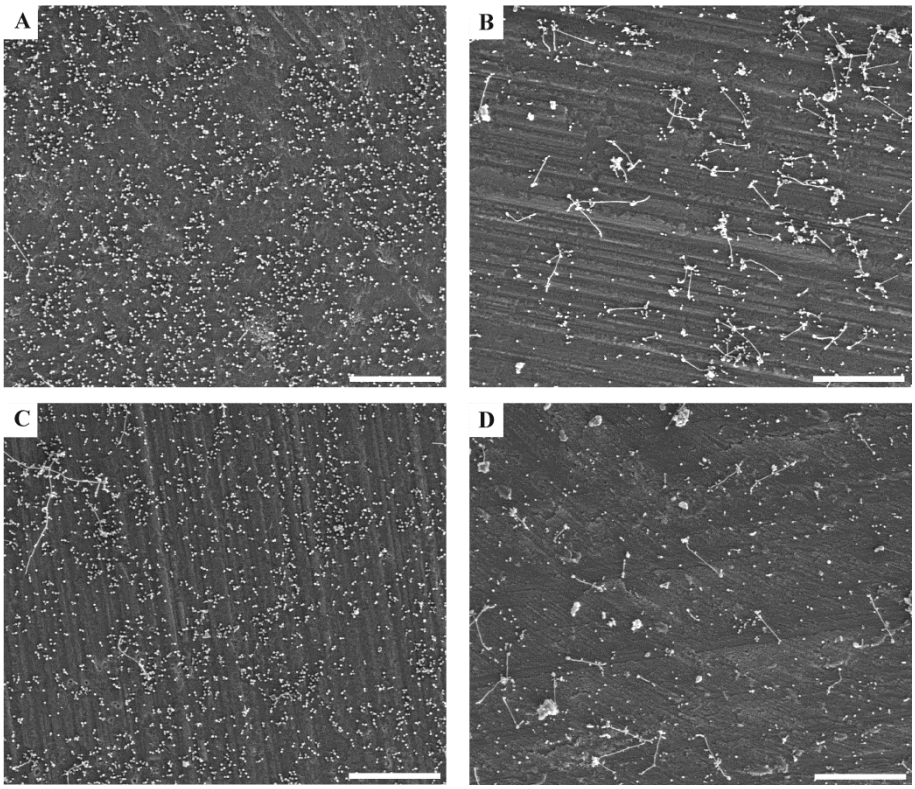

Figure 4. Morphology and spatial distribution of *C. albicans* cells on nanoHA surface after 24 h incubation with 5-aminoimidazole-4-carboxamidrazones derivatives under SEM visualization. (A) control (drug-free); (B) 2h; (C) 2k and (D) 2l compounds at 0.0; 25.0; 25.0 and 12.5 µg/mL, respectively. Magnification 500x. Scale bar 100 µm.

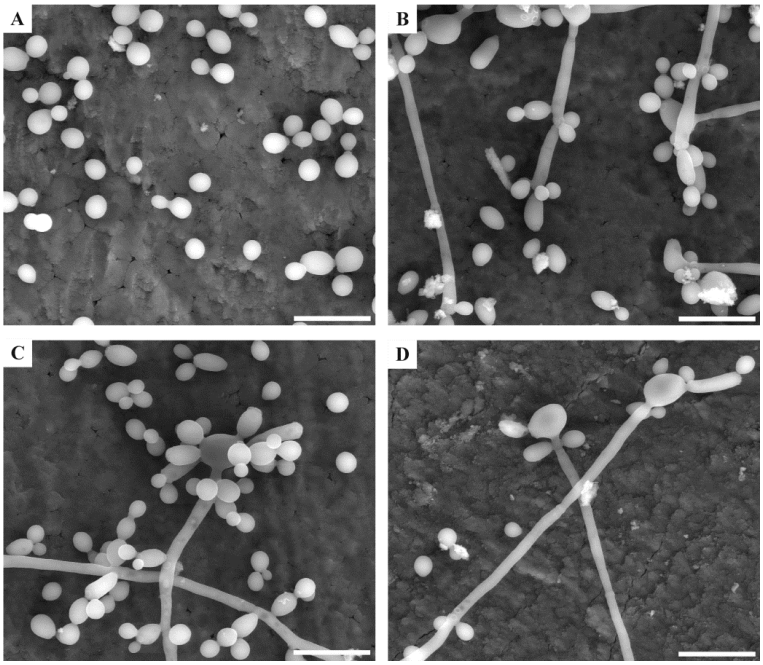

Figure 5. Morphology of *C. albicans* cells on nanoHA surface after 24 h incubation with 5-aminoimidazole-4-carboxamidrazones derivatives under SEM visualization. (A) control (drug-free); (B) 2h; (C) 2k and (D) 2l compounds at 0.0; 25.0; 25.0 and 12.5 µg/mL, respectively. Magnification 5000x. Scale bar 10 µm.

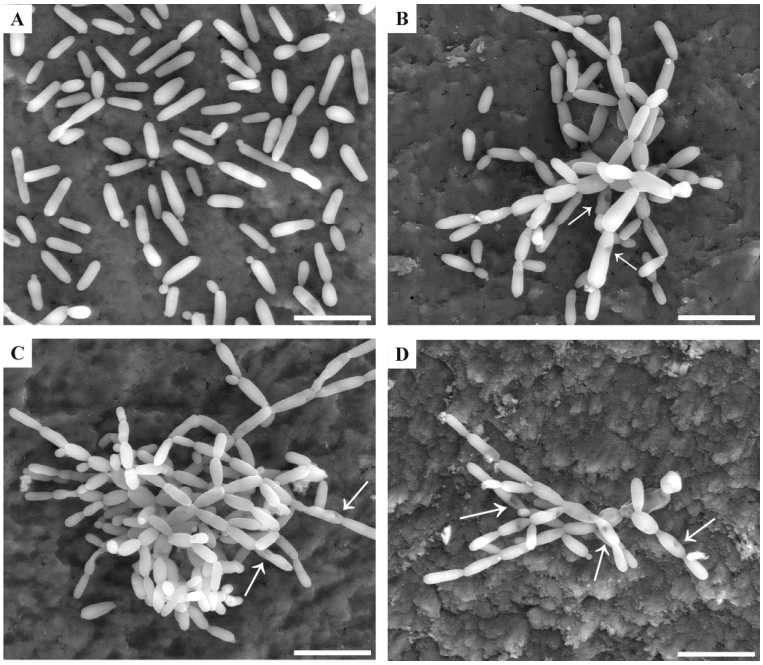

Figure 6. Morphology of *C. krusei* cells on nanoHA surface after 24 h incubation with 5-aminoimidazole-4-carboxamidrazones derivatives under SEM visualization. (A) control (drug-free); (B) 2h; (C) 2k and (D) 2l compounds at 0.0; 3.1; 3.1 and 1.6 µg/mL,

respectively. Arrows indicate the presence of disc-like depressions in cell surface. Magnification 5000 x. Scale bar 10 µm.

3.4. Cytocompatibility of 5-aminoimidazole-4-carboxamidrazones derivatives

MG-63 cells were exposed to different concentrations of imidazol derivatives that comprised all values assessed in the microbiological studies. A preliminary study had shown that the lower and the higher concentration of each compound exhibited no cytotoxicity and high cytotoxicity, respectively. For that reason, such values were excluded from the subsequent tests. Similarly to microbiological studies, two different approaches were tested. In a first approach, mammalian cells were simultaneously incubated with the compounds and the cellular metabolic activity was assessed after 24 h and 48 h of exposure (Fig. 7). Similar results were obtained for 2h and 2k compounds where 6.3 µg/mL had no cytotoxic effect after both incubation time points. However, for the subsequent concentrations, a concentration- and time-dependent effect was observed. The two highest concentrations of 2h compound, namely 25.0 and 50.0 µg/mL, proved to be highly cytotoxic. On the other hand, 2l compound presented the best cytocompatibility results, except for 25 µg/mL. Even though a time-dependent effect was noticed, the viability remained above 70% when compared to control (drug-free). Regarding the second set of experiments, the cells were allowed to adhere on nanoHA surface and, thereafter exposure to the compounds during 24 and 48 h. As depicted in figure 8, the obtained results are rather similar to the previous assay, mainly for 2k and 2l compounds. For 2h compound a sharp reduction on cellular metabolic activity after 48 h of exposure is noticed, for the lowest concentrations (6.3 and 12.5 µg/mL).

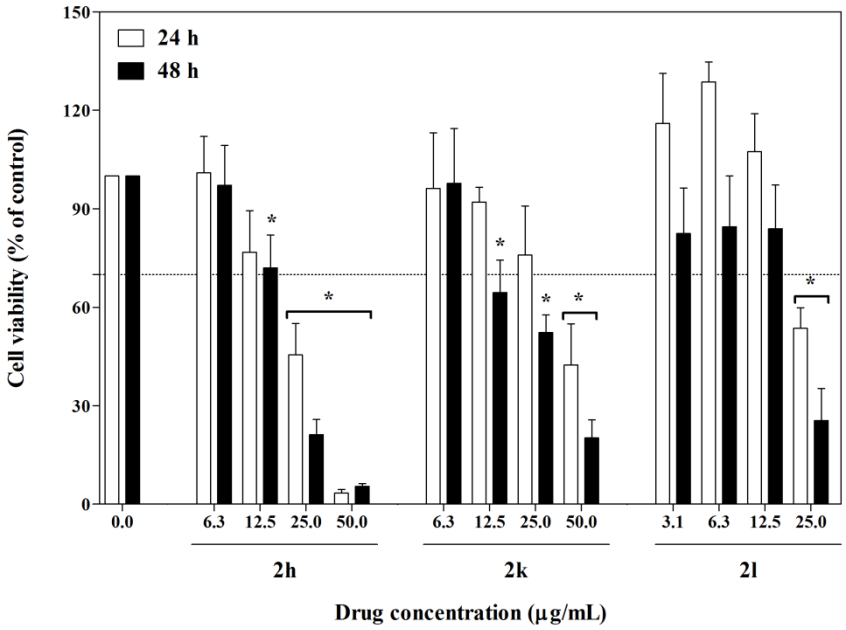

Figure 7. Effect of 5-aminoimidazole-4-carboxamidrazones derivatives on MG-63 cells metabolic activity. Cells were incubated simultaneously with different concentrations of the compounds on nanoHA surface for 24 h and 48 h. After incubation, cell viability was determined using resazurin assay. *$p < 0.05$, differences statistically significant as compared with control (drug-free samples, 0 µg/mL).

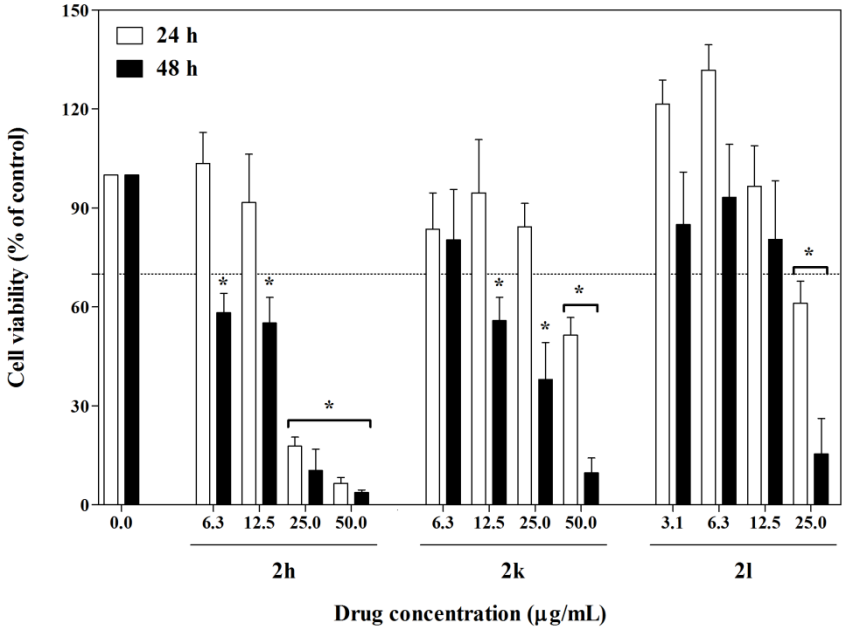

Figure 8. Effect of 5-aminoimidazole-4-carboxamidrazones derivatives on MG-63 cells metabolic activity. Cells were allowed to adhere on nanoHA substrate for 24 h and were

then treated with different concentrations of the compounds for 24 h and 48 h. After incubation, cell viability was determined using resazurin assay. *$p < 0.05$, differences statistically significant as compared with control (drug-free samples, 0 μg/mL).

4. DISCUSSION

In the present study the ability of three novel 5-aminoimidazole-4-carboxamidrazones derivatives to prevent or eliminate *C. albicans* and *C. krusei* biofilms is addressed for the first time. Both *Candida* species are causative agents of device-related infections [5, 12]. *C. krusei*, although less prevalent than *C. albicans*, has emerged as an important pathogenic, especially due to its great capacity to form biofilm [6, 11] and intrinsic resistance to fluconazole combined with reports of decreased susceptibility to both flucytosine and amphotericin B [25, 26]. Whereas the rate of successful treatment of oropharyngeal and esophageal candidiasis remains high, device-related infections by *Candida* species may represent a diagnostic and a therapeutic challenge. Even with antifungal agents currently in clinical use mortality of patients with invasive candidiasis, usually associated with indwelling medical devices, can be as high as 40% [7, 16]. Systemic administration of antifungal agents, mainly amphotericin B and fluconazole, is the 'gold standard' for the treatment of these infections, however drug therapy alone will only suppress clinical symptoms of infection at the expense of potential toxic side effects [8, 18]. One major reason for this low success rate is related with pathogenic biofilms formed on the implant surface and there appear to be multiple resistance mechanisms [4, 5, 14, 27]. *Candida* species are able to attach and form biofilm on virtually any implanted medical device in the human host, from synthetic polymers like silicone elastomer, polystyrene, polyvinyl chloride, polyurethane, to acrylic and metallic surfaces, among others [17, 28-33]. Biofilm formation studies on clinically used ceramics are yet not so abundant. Accordingly, the chosen substrate was nanoHA since is a frequently applied ceramic material for coating metallic implants or filling small bone defects in orthopaedics and dentistry, due to its high biocompatibility and bioactivity [34]. Sessile cells that are encountered in many biofilm-based device-related infections are highly resistant to the most commonly used antifungal drugs including fluconazole, caspofungin, nystatin, amphotericin B, and chlorhexidine [17,

24, 30, 35, 36]. For instance, *C. albicans* biofilms are up to 4000 times more resistant to fluconazole than their planktonic counterparts [5].

The earliest stage in *Candida* infection is the cellular attachment onto implant surfaces, often leading to biofilm development, resulting from the presence of a slimy extracellular matrix, strongly binding the cells between themselves and to the substrates. Preventing this early step is therefore critical to achieve efficient and cost-effective struggle against implanted medical devices infections. In the first part of this study, the compounds ability to restrain *C. albicans* and *C. krusei* biofilm formation, through simultaneous incubation of yeast cells and the compounds on nanoHA substrate, was evaluated. The three compounds were highly efficient in preventing *Candida* spp adhesion at concentrations equal or lower than their MICs. However, in clinical settings it's often required to act against pre-formed biofilms on implant surfaces. Consequently, in the second part of this study, the compounds effect on pre-formed *Candida* spp biofilm was evaluated. The tested compounds showed to be very effective against *C. albicans*, mostly 2k and 2l compounds. Regarding *C. krusei*, such effect against sessile population was not as strong as for *C. albicans*. For instance, the compounds exhibited low inhibition when a higher initial inoculum concentration was used. The results clearly indicate that the observed effect depends on the initial number of cells, as well as on the used species, which is consisting with other reports. Perumal *et al* [37], in a series of experiments, studied the contribution of cell density towards antifungal drug resistance in *C. albicans* biofilms and concluded that azole drug tolerance at high cell density differed mechanistically from tolerance at low cell density. Overall, *C albicans* had shown greater biofilm formation ability in both experimental approaches, which is in line with other studies [17]. Furthermore, to investigate whether the inhibitory mechanism of imidazole derivatives affected the different cellular morphologies of *C. albicans* and *C. krusei*, microscopic analysis was performed by SEM. Regarding *C. albicans*, a predominantly blastospore morphology was observed on drug-free samples. Such behaviour may be related to the hydrophilic nature of nanoHA [21]. As pointed out by other authors, the adherence of the yeast form is increased by hydrophilic coatings [38]. Moreover, it has been reported that *Candida* spp adhered more rapidly to hydrophobic surfaces due to its hydrophobic nature, which means that in the presence of a hydrophilic surface repulsive interactions take place between both surfaces and,

consequently, it could dictate the lesser biofilm network formed on nanoHA surface after 24 hours incubation [4, 39]. In this context, Frade *et al* [40] also demonstrated that *C. albicans* biofilms showed less metabolic activity on hydrophilic surfaces. Concerning *C. krusei*, the greater cell surface hydrophobicity, as determined by Samaranayake *et al* [39], might lead to a lower ability to adhere and proliferate on nanoHA, compared to *C. albicans*. After exposure to the imidazole derivatives, different effects were observed according the *Candida* species. For *C. albicans* a dimorphic transition, from yeast to filamentous growth forms, was observed. The cellular structures remained with a full shape and smooth surface, similar to those observed by Nieminen *et al* [41] in the control group. These morphological switches, as well as the overall heterogeneous morphology are common features of *C. albicans* biofilm. Filamentous growth, although not strictly essential for biofilm formation *per se*, strengthens the entire structure and provides protection and adhesion sites for the budding yeast cells, and it occurs in response to distinct environmental stimuli [16, 42, 43]. Although the substrate surface plays a role in *C. albicans* attachment, the mixture of morphological forms after exposure to the compounds strengthens the hypothesis that the chemical nature of imidazol derivatives, in particular 2h compound, could induce a phenotypic response in this species, which is a sign of cell virulence, once both hyphae and pseudohyphae might promote tissue invasion during the early stages of infection [43]. On the other hand, *C. krusei* exhibited its 'large American rice grain' form devoid of hyphal elements as also observed by Parahitiyawa *et al* [11]. The exposure to the imidazole derivatives seems to have induced the formation of microcolonies on nanoHA surface. The majority of cell surfaces were smooth and free of wrinkles, albeit some of them presented disc-like depressions, in accordance to that recently reported by Ma *et al* [44]. Sample preparation for SEM usually requires an approach that subjects samples to mechanical and physical stress and can alter normal biofilm morphology, however such artefacts might be dismissed in the present study since only localized cell damage were seen, most likely induced by the imidazol derivatives. As suggested by Ma *et al* [44], and in line with our results, such morphological changes may indicate that the antifungal effect was the result of a loss of integrity of the cell surface and changes in cell membrane permeability. The morphological alterations associated to each specific *Candida* species underline that different effects are occurring. This is one of the reasons

why it was considered relevant to introduce in this study the use of two different *Candida* species instead of only one, often *C. albicans*, because, as observed, major differences between species with respect to susceptibility profiles and the ability to adhere to biomaterial surfaces were identified, even so this study would benefit from confirmation with further *Candida* species and clinical isolates due to inter-strain variations.

A especially important characteristic of all biofilms is the presence and composition of the extracellular matrix that serves as a diffusion barrier to antifungal agents [4]. However, in the present study both *Candida* species were apparently devoid of extracellular polymeric material covering the fungal microcolonies. Similar evidences were reported by Samaranayake *et al* [45]. The matrix is usually difficult to preserve for SEM observation. However, the presence of *Candida* matrix depends not only on preparative techniques but also on the incubation conditions during biofilm development [2]. The absence of matrix might partly explain the high sensitivity of *Candida* spp to the imidazole derivatives. Regarding the cytocompatibility assays, parallel results were found between the same compounds, for both approaches. Which means that independently from time of compounds administration, a time-dependent cytotoxicity is noticed. By combining the results of cytocompatibility and antifungal activity, it may be observed that 2h compound showed the less appealing results once the majority of *C. albicans* cells showed dimorphic transition and a significant reduction in the number of remaining viable cells was only observed for concentrations as high as the MIC (50 µg/mL) for the pre-formed biofilm experiment with the highest inoculum concentration tested (Fig. 3), for which high cytotoxicity was observed (Figs. 6 and 7). On the contrary, 2l proved to be the most effective compound for both *C. albicans* and *C. krusei* for the lowest concentrations tested 12.5 and 1.6 µg/mL, respectively, with no cytotoxic effect against MG-63 cell line.

5. CONCLUSION

The three tested 5-aminoimidazole-4-carboxamidrazones derivatives, in particular the 2l compound, have shown to present promising therapeutic potential to hamper biofilm-based device-related infections caused by pathogenic fungi since they display powerful *in vitro* activity against *C. albicans* and *C. krusei* biofilm formation and pre-formed

biofilms, that have shown to be otherwise refractory to treatment with the most commonly used antifungal agents. Additionally, the antifungal resistance of biofilm-grown cells increased in conjunction with biofilm formation therefore, a winning strategy shall need to address the first moments of yeast adhesion, as preventive measure. Furthermore, 2l compound exhibited the best cell viability values. Summing-up all the obtained results, the 2l compound ought to be further investigated as a potentially reliable antifungal agent for local therapy.

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CHAPTER VI

General discussion and future perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In 1973, Andriole was among the first researchers to realize that foreign bodies could potentiate the development of osteomyelitis [1]. Since then, a long way has been covered both to understand the biological principles behind such clinical events and to design strategies to avoid them. At present, there is not a single strategy that could totally eliminate the incidence of infections associated to biomaterials. Microbial adhesion and subsequent biofilm formation are major threats to human health as they are inherently resistant to clearance by both the host immune system and antibiotics [2, 3]. Engineered implants to prevent infection are therefore a bubbling field with a wide range of strategies being explored to impair microbial adhesion. In particular, tissue engineered scaffolds for bone applications provide a convenient framework for tissue repair, as well as a substrate for the inclusion of antimicrobial agents, to prevent and treat infections after surgical intervention. Introducing anti-infection properties into regenerative medicine therapies could improve clinical outcomes and reduce the morbidity and mortality associated with biomaterial implant-related infections.

Synthetic nanoHA is a beneficial material for medical applications due to good physicochemical similarity with bone [4], which opens up opportunities for applications throughout the body, for instance as a local delivery system of antibiotics. Although this type of approach has generally shown favourable results regarding their efficacy, there are a number of challenges which may slow down its clinical application, above all the emergence of new antibiotic resistant clinical strains [5, 6]. Therefore, finding treatments that can alter the phenotype of the bacteria without inducing and selecting for genetic modifications that could lead to resistance is a key factor in winning the battle against these pathogens. One strategy explored in this work was the use of ZnO due to its well-known antimicrobial activity [7]. In comparison with organic agents, the use of inorganic compounds, such as ZnO, for the production of nanoHA-ZnO composites has several practical advantages. They are more robust, resist to harsh process conditions and are capable of being synthesized in large quantities with a desired composition, reproducible size and structure. Their higher durability and stability promote a long-term shelf-life, which is a key condition for the use of a biomaterial in clinical settings. Furthermore, this experimental strategy does not lead to emergence of resistant bacteria; it may mitigate concerns about multi-drug resistant super-bugs commonly seen in

conjunction with approaches based on conventional antibiotics. A step ahead, three-dimensional and interconnected nanostructured porous granules of HA incorporated with small amounts of ZnO nanoparticles were produced and the resulting biomaterials supported tissue recovery, with the presence of new blood vessels and tissue regeneration *in vivo* and protected the biomaterial surfaces from biofilm formation not only *in vitro* but also *in vivo*. Regarding the organic agents, not everything is weakness. For instance, propolis can be by itself a bioactive product or be a promising source of new bioactive compounds. Additionally, it poses low risk for the development of resistance and presents high bioavailability [8]. A last approach addressed in this work was the use of new imidazole-based components to act against *Candida* spp biofilm. The preliminary results obtained showed promising therapeutic potential of these components to be used either as preventive measure or treatment procedure in the case of biomaterial-related infections by fungi.

Beside the aforementioned approaches, which were successfully tested, the development of surfaces with multiple functionalities is therefore a new challenge for biomaterials engineering. Such surfaces should combine the need for implants possessing anti-infective properties with much needed maintenance of peri-operative tissue homeostasis. Under these circumstances multifunctional surfaces may give host cells a leg up in winning the race for the surface [9], as opposed to often-reported mono-functional surface chemistries and morphologies that either discourage microbial adhesion and biofilm growth or promote host tissue integration but cannot reach both simultaneously [10]. To achieve such goal, Muszanska *et al* [11] work describes the synthesis and characterization of a polymer-peptide conjugates to be used as infection-resistant coating for biomaterial implants. Anti-adhesive polymer brushes composed of block copolymer Pluronic F-127 were functionalized with AMP, being able to kill bacteria on contact, and arginine-glycine-aspartate acid (RGD) peptides to promote the adhesion and spreading of host tissue cells. The novel bioactive surfaces showed good anti-adhesive and bactericidal properties against *S. aureus*, *S. epidermidis* and *P. aeruginosa*, without hampering tissue compatibility. Another example of such approach is the work developed by Pishbin and colleagues [12] which tested multi-functionalizing chitosan-based composite coatings involving addition of bioactive glass particles and gentamicin as a molecular antibacterial agent. The coatings showed to be bioactive and

the gentamicin release kinetics could inhibit bacterial growth for the first 2 days and could support cellular proliferation for up to 10 days. Several others approaches for multifunctional surfaces have been proposed and tested [13-16], with very promising results. Nonetheless, they need to fulfil some practical requirements namely: they should be easily applicable to the surface of a large number of (ingrowth and non-ingrowth) implants, have a longer-term effectiveness, demonstrate no local and systemic toxicity and be cost-effective.

In the near future, it might be possible to develop multifunctional, self-responsive, and self-repairing biomaterials refereed as 'smart coatings'. These coatings are designed to be sensitive to various external and internal stimuli, thereby enhancing the surface functionality of materials. It is anticipated that these coatings respond intelligently to signalling depending on how they are prepared. Accordingly, smart coatings should possess synergistic passive and active functionalities [17, 18]. The most sophisticated part of smart coatings is the development of nanocontainers with sensitive shells, high loading capacity, and good affinity for the coating matrix. The sensor unit is also a critical component of a smart micro-device that should be sensitive enough to detect even the earliest microbial-associated signals. For instance, nanocontainer shell entryway signals include pH, temperature, as well as mechanical, chemical, and electrical (electrochemical) changes in the peri-implant effective space. Stimulation should then lead to the opening of the nanocontainers and release of specific substance(s) [17, 19, 20]. For example, nanopatterned poly(N-isopropylacrylamide)/ quaternary ammonium salt hybrid surfaces are model systems that exhibit an ability to undergo non-covalent, dynamic, and reversible changes in structure that can be used to control the attachment, killing, and release of bacteria in response to changes in temperature [21]. Some of the challenges encountered during the development of these smart coatings have included: survivorship during the implant-coating manufacturing process, non-adverse reactions to the smart coatings themselves *in vivo*, mechanical resistance, and preservation of intended functionalities throughout the device's expected and functional life time [17, 22].

The critical aspect for the effective translation of novel biomaterials from the laboratory to the clinic is the current commonly used pre-clinical validation methods, either *in vitro* or *in vivo*. There is no widely accepted methodology available that could

precisely and reproducibly demonstrate antimicrobial behaviour of the proposed anti-infective approaches. Controllable, standardized methods, in particular to test biofilm-growing bacteria, must be improved, to more accurately reflect the clinical situation, including the influence of physiologically relevant conditioning films, the use of relevant pathogenic species and the host response. Therefore, a more precise prediction of biomaterial clinical success could be expected. Examination of published studies on this topic suggests a striking discrepancy between proposed strategies of antibacterial surface treatment and ultimate completion of *in vitro* and *in vivo* experimentation. Barriers to translational medicine in this field are most likely related to economic, medico-legal, and biotechnological issues. Concerns about the long-term durability of such new implantable devices as compared to traditional implants are also realistic. Only by improving collaborative efforts between governments, regulatory agencies, industry leaders and health care players will patients benefit from these technologies [22, 23].

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